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AN INVESTIGATION INTO THE BIOCONVERSION OF SOUTH AFRICAN SUB-BITUMINOUS COAL BY *TRAMETES* SPECIES AND *TRICHODERMA ATROVIRIDE*

BY

CARYN J. VENGADAJELLUM

Thesis submitted for the degree of DOCTOR OF PHILOSOPHY in the
Department of Chemical Engineering
Biocatalysis and Technical Biology Research Group
UNIVERSITY OF CAPE TOWN
December 2008

ACKNOWLEDGEMENTS

I would like to acknowledge Professor Stephanie Burton for the supervision and direction she willingly gave me during this project. We have had a long haul together and I have gained invaluable experience from her along the way. I would also like to thank Professor Sue Harrison and Professor Don Cowan for the support, advice and technical expertise they gave during this collaborative project. Thank you to Steven Makhonghela, Frans Ngako, Bilanu Oboirien, Hussnain Janjua and Estela Silva-Stenico for their contributions to this work.

Thank you to Piero Benincasa of the Chemistry Department (elemental analysis), Miranda Waldron of the EM unit (electron microscopy), Helen Divey of the Chemical Engineering analytical laboratory and the staff of the Chemical Engineering workshop for their technical assistance. My deep gratitude goes to my friends and colleagues in the Department of Chemical Engineering for fostering a warm but professional environment in which to study. I would like to specially thank Sue Jobson and Frances Pocock, for their dear friendship, kind words, shared laughter and tears, wealth of knowledge and wisdom, and their invaluable support which saw me through some very difficult times during this project. I salute you! Thank you to Dr Clive Garcin for helping me with the airlift bioreactors and for proof-reading this thesis. Madelyn Johnstone-Robertson, Thanos Kotsiopoulos and Alistair Hughes, thank you for helping me with stoichiometry, mass balances and MATLAB.

I gratefully acknowledge the National Research Foundation and the University of Cape Town for their financial support.

Thank you to all those (past and present) who meet at 43 Alpina Road, Claremont, every Thursday evening. Without your constant support, encouragement and prayer, I would not have endured. Timothy – I'm lost for words! Thank you for listening and carrying the burden with me even when you couldn't understand. Your companionship and friendship helped me to persevere when giving up seemed so much easier.

My most heartfelt gratitude goes to my family, who have all been very patient. Mummy and Daddy – thank you for being the greatest examples of Christ. Your unconditional love and support has given me the freedom and courage to pursue my dreams. Thank you for your selfless giving and sacrificial love. Dee and Vern- thank you for pushing me, and carrying me at times, and for all the “financial aid”.

Lastly, all praise and honour belong to God, who lifted me up and never let me go when everything seemed to fail. Its all for His glory!

"Whatever is true, whatever is noble, whatever is right, whatever is pure, whatever is lovely, whatever is admirable, if anything be excellent or praiseworthy, think about such things"

Philippians 4 vs 8

*In loving memory of Ethel Rosa Birkholtz
(1912-2008)*

*Dedicated to Mr and Mrs R.F. Vengadajellum, in honour and celebration of their Golden wedding
anniversary (26 December 2008)*

ABSTRACT

The development of coal utilisation processes is currently being pursued globally due to the increase in oil price, demand for liquid fuels, and energy security concerns. Apart from the production of liquid fuels, there is a renewed interest in utilising the aromatic content of coal as a resource for the production of chemical derivatives. The biosolubilisation (solubilisation using microbial processes) of coal is an attractive alternative to the traditional thermal derivation of coal liquefaction products due to the environmentally benign conditions associated with it. Thus, the biosolubilisation of coal could be an important starting point for the production of feedstocks for both fuel and chemical industries.

The work presented in this thesis describes the preliminary investigation of the bioconversion of a South African (SA) sub-bituminous coal to produce low molecular mass intermediates which may be used in further processes to yield value-added compounds. Low rank coals (LRC), which are less desirable for combustion processes than high rank coals, are more amenable to biodegradation due to their high moisture content and oxygen-containing functionalities. LRC are therefore ideal for application to biological processing. Microorganisms that are capable of modifying the coal structure by different mechanisms have been identified. Among these are the well-characterised lignin-degrading fungal systems, which have been commonly investigated for the bioconversion of LRC due to the lignino-cellulosic origin of LRC, and thus its structural similarities to lignin.

The bioconversion of coal to yield value added products is still at the fundamental research stage and has focused mainly on the biosolubilisation of low rank coals such as lignite. There is still a need to discover the ideal combination of coal type and microbial strain. This includes the systematic screening of microorganisms for coal bioconversion activity and the determination of biochemical processes and metabolic pathways they employ. The characterisation of catabolic pathways generated by enzymatic attack on coal is required, as well as the determination of the identity and fate of catabolites. Furthermore, appropriate reactor designs for the bioconversion of coal have not been extensively researched yet. The aim of this study was, thus, to address some of these key issues.

The first objective of the work was to characterise the SA sub-bituminous coal through proximate and elemental analysis to determine the chemical and physical properties of the coal. The next objective was to identify microbial strains that could effectively breakdown the carbon matrix of coal through initial biosolubilisation and subsequent depolymerisation reactions to yield compounds of lower molecular mass, and to identify the enzymes responsible for coal bioconversion and the major products released. Finally, an appropriate bioreactor design for the bioconversion of coal by the selected strain was investigated. The kinetic data generated from this system would be the basis for preliminary calculations of the process yields for the bioconversion of coal.

Analysis of the coal used in this work showed that the carbon content was lower than that expected of a sub-bituminous coal. The high volatile content gave an indication of the aromatic carbon compounds present in the mobile phase of the coal, which could support growth of microorganisms. A reliable indicator of the relative susceptibility of coal to microbial entry and subsequent degradation is given by the internal porosity of the coal, which is a measure of the inherent moisture content. A low inherent moisture content was measured, which suggested that this coal may be more recalcitrant to microbial attack than expected.

A solid media screening system was successfully used for the identification of fungi with the potential to degrade low rank coal. The fungal strains were obtained from a selection of environmental isolates (isolated from soil, wood and coal) and international culture collections. Evaluation of the selected fungal strains was based on the ability of these strains to produce extracellular, ligninolytic enzymes (capable of C-C and C-O cleavage), depolymerise coal macromolecules, and to grow on coal model compounds as a sole carbon source. *Trametes versicolor* and *Trametes pubescens* were the best performers among the 13 fungal strains tested. In addition to the *Trametes* spp., experiments in liquid culture included *T. atroviride* ES11 based on its ability to grow on coal model compounds, but primarily owing to the indigenous origin of the strain and few reports describing its use in the bioconversion of coal.

T. atroviride ES11 was able to degrade the SA sub-bituminous coal in slurry systems but the diversity of compounds produced was limited. 4-Hydroxyphenyl ethanol, 1, 2-benzenediol and 2-octenoic acid, identified by GC-MS, were the only compounds recovered following the bioconversion of coal by *T. atroviride* ES11. The bioconversion of coal only occurred in the presence of actively growing mycelia and/or its extracellular products. Although extracellular esterases and oxidases have been implicated in coal bioconversion by other *T. atroviride* strains, none were detected in shake flask experiments carried out in this study. However, a number of intracellular dehydrogenase enzymes, involved in aromatic ring cleavage, were detected and some activities were induced in the presence of coal. The close physical association between coal particles and the fungal mycelia, together with the adhesion of intermediate metabolites, was perceived to have facilitated the metabolism of coal degradation products to form biomass and carbon dioxide. An effective carbon mass balance could not be constructed due to the adhesion of coal particles to fungal hyphae but work done in a collaborative study later confirmed that the primary fate of coal products was biomass and carbon dioxide.

Because coal macromolecules are too large to be taken up into the cell, an extracellular mode of attack is required to break it down into lower molecular mass compounds. Since no clear extracellular mechanisms were identified in *T. atroviride*, the focus of this study changed to investigating the bioconversion of coal by the *Trametes* spp. which were identified in the solid media screening system. The bioconversion of SA sub-bituminous coal, lignite and asphaltene (hydrogenation product of hard coal) by *T. versicolor* and/or *T. pubescens* was demonstrated in shake flasks. The biosolubilisation of coal resulted in the production of

humic acids which corresponded to high activity of laccase, an extracellular oxidase enzyme, by the fungi. A complex relationship between laccase activity and its inhibition by the humic acids produced was highlighted. In addition to this, the presence of fungal biomass and the adhesion of coal particles to fungal hyphae (as also observed with *T. atroviride*) lead to mineralization of intermediate products and also complicated the quantification of coal degradation by gravimetric analysis. Elemental analysis of coal residues, subjected to acid digestion to remove fungal hyphae, showed that 11.45 and 9.45 % coal carbon was degraded by *T. versicolor* and *T. pubescens*, respectively. This was an important result for the bioconversion of sub-bituminous coal since similar values reported in literature only correspond to the bioconversion of lignite, which is more amenable to biodegradation than sub-bituminous coal. In an attempt to delineate this complex system, the bioconversion of coal by cell-free extracts of *T. pubescens* was investigated and showed that coal macromolecules were depolymerised in the absence of fungal biomass, resulting in compounds of lower molecular mass. 9.7% coal carbon was converted by cell-free extracts of *T. pubescens*.

The yields of reported processes producing biosolubilised coal and coal catabolites are low, and kinetic analysis of coal bioconversion has not been sufficiently reported in literature. Thus, the final objective of this work was to investigate suitable bioreactor configurations for the bioconversion of coal by *T. pubescens*. The draught-tube, internal loop airlift bioreactor proved to be best bioreactor for this application amongst four configurations tested (stirred tank, fluidised-bed, packed-bed and airlift bioreactors) possibly due to the increase mass and oxygen transfer. A black-box approach to balance stoichiometric equations and an indirect method of biomass determination were used to evaluate the kinetics and product yields of the bioconversion of coal by *T. pubescens*. Coal conversion of 20.9% was achieved in the airlift bioreactor with biomass and carbon dioxide being the major products, and small amounts of humic acids.

To the best of the author's knowledge, this was the first report on the stoichiometric analysis of the bioconversion of coal using a white-rot fungus and the first research project into the bioconversion of a SA sub-bituminous coal by *T. pubescens*. Before the applied side of coal bioconversion can progress, further fundamental knowledge of the biochemical pathways is required. Recommendations were made in several directions, but most important would be the pursuit of white rot fungal systems for the bioconversion of coal and the development of a system that prevents mineralization of intermediate products. Such a system could allow for the continuous removal of products or incorporate a two-step process where biomass production and regeneration of extracellular enzymes and co-factors occur separately to the contacting of coal with the cell-free extract. This would be advantageous for the accumulation of low molecular mass aromatic compounds such as 4-hydroxyphenyl ethanol (tyrosol) and 1, 2-benzenediol (catechol), which have commercial value.

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LIST OF ABBREVIATIONS

A _{450NM}	Absorbance at 450 nm
ABTS	2,2' –azinobis-(3-ethylbenzathiazoline-6-sulphonic acid)
ALR	Airlift loop bioreactor
ARCAM	Advanced Research Centre for Applied Microbiology
CBS	Centraalbureau voor Schimmelcultures
CER	Carbon dioxide evolution rate
CSA	Coal solubilising agent
CSTR	Continuous stirred tank reactor
DMAB	3-Dimethylaminobenzoic acid
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
DO	Dissolved oxygen
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	Ethylene diamine tetra-acetic acid
FBR	Fluidised-bed bioreactor
GC/GC-MS	Gas chromatography/ gas chromatography – mass spectrometry
1-HBT	1-Hydroxybenzotriazole
HPLC	High performance liquid chromatography
HPLC-SEC	High performance liquid chromatography- size exclusion chromatography
IR	Infrared spectroscopy
KDa	Kilo daltons
LiP	Lignin peroxidase
LRC	Low rank coal
MBTH	3-Methyl-2-benzothiazolinone hydrazone
MnP	Manganese peroxidase
NMR	Nuclear magnetic resonance
OUR	Oxygen utilisation rate
PBR	Packed-bed bioreactor
PHA	Polyhydroxyalkanoates
PAH	Polycyclic aromatic hydrocarbon
PPRI	Plant Protection Research Institute
RBBR	Remazol Brilliant Blue R
RDR	Rotating drum reactor
SA /RSA	South African/ Republic of South Africa
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM	Scanning electron microscope (microscopy)
SmF	Submerged fermentation
SSF	Solid state fermentation
STR	Stirred tank bioreactor
TDM	Trametes defined media
THF	Tetrahydrofuran
TOC	Total organic carbon
UV-Visible	Ultraviolet-Visible
WRF	White rot fungi

University of Cape Town

OUTPUTS ARISING FROM THIS WORK

Published articles:

Silva-Stenico, M.; Vengadajellum, C.J.; Janjua, H.A.; Harrison, S.T.L.; Burton, S.G. and Cowan, D.A. (2007) Degradation of low rank coal by *Trichoderma atroviride* ES 11. *J. Ind. Microbiol. Biotechnol* **34**(9): 625-631

Oboirien, B.O.; Vengadajellum, C.J.; Burton, S.G.; Cowan, D.A and Harrison, S.T.L. (2008) The effect of the particulate phase on coal biosolubilisation mediated by *Trichoderma atroviride* in a slurry bioreactor. *Fuel Proc Technol* **89**:123-130 *

Kirby, B.M.; Vengadajellum, C.J.; Burton, S.G and Cowan, D.A. (2008) Anthropogenically-created habitats – coal, coal mines and slag heaps. In Handbook of hydrocarbon microbiology: Microbial interactions with hydrocarbons, oils, fats and related hydrophobic substrates and products. Kenneth N. Timmis (ed)

Conference presentations:

C. J. Vengadajellum; M. E. Silva-Stenico; S. T. L. Harrison; D. A. Cowan; S. G. Burton. Bioreactor systems for biosolubilisation of low rank coal. International Biotechnology Symposium, Santiago, Chile, 2004

M. Estela. Silva-Stenico; Caryn. J. Vengadajellum; Stephanie. G. Burton; Sue. T. L. Harrison; Marshall Solomon; Donald. A. Cowan. Microbial and enzymic solubilisation of low rank coal. International Biotechnology Symposium, Santiago, Chile, 2004

C.J. Vengadajellum; S.T.L. Harrison; D.A. Cowan; M.S. Solomon; and S.G. Burton. Biosolubilisation of low rank coal: a novel route to value added compounds. SASBMB National conference, Pretoria, South Africa, 2003

C.J. Vengadajellum; S.T.L. Harrison; M. Solomon; D.A. Cowan; S.G. Burton. Biosolubilisation of low rank coal: a novel route to value added compounds. South African Institute of Chemical Engineers, R&D day, University of Stellenbosch, Stellenbosch, South Africa, 2003

* See Appendix D

C.J. Vengadajellum; S.T.L. Harrison; M. Solomon; D.A. Cowan; S.G. Burton. Biosolubilisation of low rank coal: a novel route to value added compounds. Cape Biotech Initiative conference, Somerset West, South Africa, 2002

Provisional patent application:

C.J. Vengadajellum and S.G. Burton (2008) Asphaltene biodegradation Provisional patent no. ZA 2008/05093. South African Patent Office.

Articles in preparation:

Vengadajellum, C.J.; Harrison, S.T.L.; Cowan, D.A. and Burton, S.G. (2008) Depolymerisation of low rank coal macromolecules by a cell-free preparation of *Trametes pubescens*. In prep.

Vengadajellum, C.J.; Harrison, S.T.L.; Cowan, D.A. and Burton, S.G. (2008) Kinetic analysis of the bioconversion of sub-bituminous coal by *Trametes pubescens*. In prep.

CHAPTER 1: LITERATURE REVIEW AND RESEARCH OVERVIEW

1. 1 INTRODUCTION

This thesis describes the use of filamentous fungi and/or their enzymes in the bioconversion of low rank coal (LRC) with the aim of producing value-added compounds. South Africa leads the southern African countries and is also ranked fifth in the world, in terms of coal production and export (Cairncross, 2001), with sub-bituminous coal being the major product. Approximately 74% of South Africa's energy usage is coal-based, this being the main source of energy in southern Africa. The abundance of LRC and its unsuitability for direct use as a fuel offers a unique opportunity for value-addition to this natural resource. The conventional thermo-chemical methods of solubilising coal are energy intensive and expensive since operating conditions are extreme (involving high temperatures, pressures and harsh chemical environment). This, coupled with an increasing awareness of threats to the global environment, has resulted in a movement towards the development of "green" technologies and "Clean Coal Technologies" (Fakoussa and Hofrichter, 1999). New and environmentally acceptable technologies for the effective utilisation of coal as an energy source and chemical feedstock are currently being developed, prompted by the rapidly dwindling supply of oil and natural gas and the recent substantial increases in oil prices (Tullo and Tremblay, 2008).

One approach is the utilization of biotechnological processes to convert coal to a clean, cost-effective energy source or to value-added products (Fakoussa and Hofrichter, 1999). This includes the biosolubilisation of coal, a process involving the microbial conversion of solid particles, which results in the dissolution of coal macromolecules in an aqueous liquid. The advantages of coal biosolubilisation would include:

- Simplification of mining, transport and handling of coal.
- Reduced operating costs and lower environmental burden due to the milder operating conditions associated with biological processes.
- Increased coal recoverability as a result of *in situ* transformation of coal within the coal seam.
- Increased versatility of coal as fuel through the conversion of coal to fuel alcohols or alkanes using traditional fermentations (Faison, 1993).

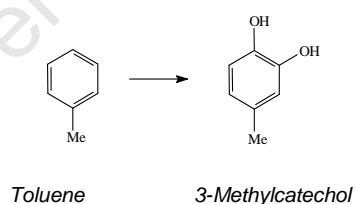
Biosolubilised coal has greater bio-availability and bio-accessibility than that obtained by chemical solubilisation methods, thus widening the scope of its applications to biogasification, biodesulphurisation and biotransformation processes. In *biogasification*, aqueous products from coal biosolubilisation could be considered as feed material for further bioprocessing steps that employ mixed cultures of anaerobic bacteria to convert coal liquids to methane (Scott, 1990).

The biosolubilisation of coal could increase the accessibility of organically-bound sulphur to sulphur-oxidising microorganisms in the *biodesulphurisation* of coal (Klein *et al.*, 1988). This process is necessary to eliminate the sulphur dioxide emissions into the environment when coal is used as a fuel (Baek *et al.*, 2002), a factor that is particularly prevalent with burning of low rank coals such as lignite, due to its high sulphur content (Gokcay *et al.*, 2001).

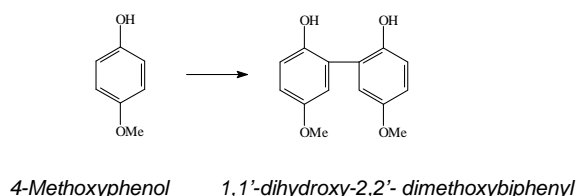
The biosolubilisation of coal does not necessarily include the depolymerisation of coal macromolecules (which is the catabolism to units of lower molecular mass), but depolymerisation is unlikely to occur unless biosolubilisation has first occurred. Thus, biosolubilised coal may consist of a mixture of polar organic compounds with moderate to high molecular weights and a high degree of aromaticity, and would, therefore, be a potentially useful starting material for more aggressive biological treatments designed to yield value-added products (Scott *et al.*, 1986; Ralph and Catchside, 1996). Achievable targets from the *biotransformation* of biosolubilised (and biodepolymerised) low rank coal include the production of oligophenolics, naphthols, nutraceutical antioxidants and other chemical derivatives. For example, peroxidases have been demonstrated to have the ability to oxidise toluenes to aldehydes, which are often used as feedstocks in industrial chemistry (Russ *et al.*, 2001). Hence, certain substituted derivatives of toluene, which are products of coal biosolubilisation, can be further biotransformed into their corresponding aldehydes.

Other examples of biotransformations of coal constituents (see Table 1.1 on page 8) include:

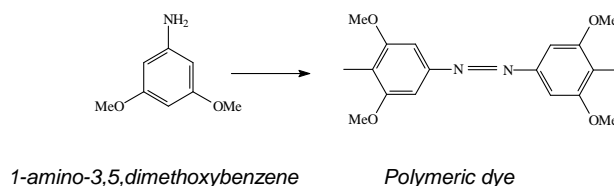
- Methylbenzenes to catechols (Burton, 1994)



- Phenols to dimers and polymers (Setala *et al.*, 1994)



- Aromatics to dyes (Vasquez-Duhalt *et al.*, 1995)



Several bacteria have also been identified that can use chemically heterogeneous, biosolubilised coal as a complex carbon source for growth. For example, *Pseudomonas oleovorans* and *Rhodococcus ruber* were able to accumulate polyhydroxyalkanoates (PHAs) when the cells were cultivated on coal biosolubilisation products in the absence of any other carbon source (Fuchtenbusch and Steinbuchel, 1999). Hence, there is potential for the production of PHA's from coal biosolubilisation products.

1.2 COAL

1.2.1 Coal origin and composition

Coal is a complex heterogeneous mixture of macromolecular organic compounds with an irregular chemical structure. The formation of coal involves rearrangement of organic and inorganic chemical constituents (diagenesis) and metamorphism, resulting in a compact and highly crystalline condition (Faison, 1993). The organic component of coal originates mainly from plant material, and evolution of different plant types has influenced the composition of the coals formed (Elliott, 1981; Faison, 1993). The initial reactions of coal formation involve the decay of dead plant tissue by microorganisms. Peat, the progenitor of coal, is formed as the result of the incomplete degradation of plant tissue, and consists of fibrous organic material enriched with humic acids with a very high moisture content. The microbial degradation of peat ceases as inorganic sediments are gradually deposited over the organic layer containing the decayed plant material and entrapped microbial biomass. Coal formation (depicted in Figure 1.1) results as the heterogeneous organic matrix undergoes the metamorphic process, which is driven by changes in temperature (due to climate and geothermal activity), pressure and geological time effects (Faison, 1993).

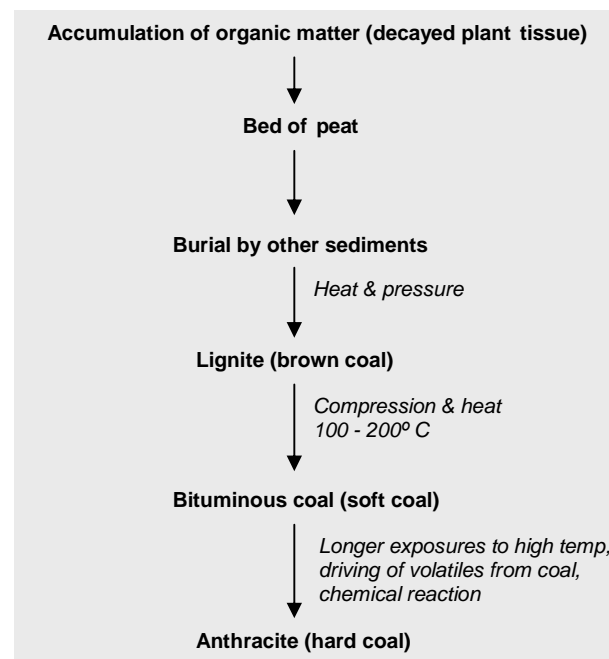


Figure 1.1 The coal formation process (Morris, 2000)

1.2.2 Coal classification

Coal consists of three components, namely the organic component, the mineral component and the moisture content. Each component is altered as coalification proceeds. The rank of a coal is indicative of how far along the coalification path the coal has progressed (Klein *et al.*, 1988), starting from peat, through lignite (lowest rank), sub-bituminous coal, bituminous coal and anthracite (highest rank). Increases in the aromatic carbon content and aromatic ring condensation are characteristic of higher rank coals. Thus, anthracite, containing the most highly condensed aromatic ring structure and the highest carbon content, is the highest ranked coal (Faison, 1993).

Coals from different seams, coalfields and regions of the world vary greatly in terms of origin, nature, constitution, and maturity (Falcon and Ham, 1988). Southern African coals and those from other Gondwana provinces (India, Australia and South America), when compared with coals from the northern hemisphere, differ in that they are rich in minerals, relatively difficult to beneficiate, and possess high variability in rank and organic-matter composition. These differences are attributed to the conditions at the time of formation and the corresponding geological events over time (Falcon and Ham, 1988). Such variation leads to classification of coal in terms of coal types (organic composition), grades (mineral-matter composition) and rank (maturity). Thus, analysis of coals on the empirical level and the fundamental level can provide information of qualitative, technological, and economic significance (Figure 1.2).

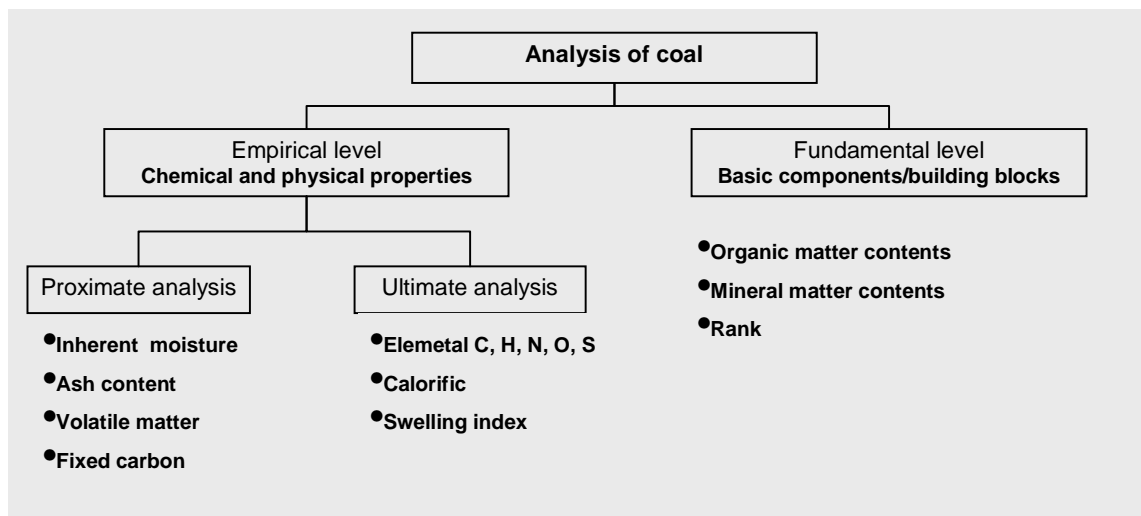


Figure 1.2 Description of coal analysis on an empirical and fundamental level (adapted from Falcon and Ham, 1988)

The major difference between European and North American coals, as compared with Southern African and other Gondwana countries' coals, is the range of rank. The latter group includes lignites, sub-bituminous and low-rank bituminous coals, which are particularly reactive to oxidation and combustion, based on their inherent properties. The bulk of the former group coals lie in the mid to low volatile, bituminous range grading to anthracite (Falcon and Ham, 1988).

South African coal reserves are relatively large, mainly due to the existence of the Karoo basin. The coals range from sub-bituminous to mid-bituminous, although anthracite does occur in eastern regions. There is a trend across the South African coalfields, from east to west, of an increase in rank (Cairncross, 2001), possibly caused by greater geothermal gradients in the east where the lithosphere was relatively thin. Lignite deposits, however, are limited, in contrast to the large bituminous reserves that are concentrated in the northern half of South Africa (Jacobs and Cole, 1999).

1.2.3 Chemical properties of coal

Low rank coals tend to be younger than high rank coals and are typified by high inherent moisture contents, high volatile contents, low carbon contents and high oxygen content. Lignite, also referred to as brown coal, bears less condensed aromatic structures and more ether linkages and methoxy residues, compared to bituminous coal. Consequently, lignite is much more hydrophilic in character, and is more porous in structure, than higher-ranking coals. A comparison of coals of different ranks by infrared (IR) spectroscopy indicated that lower rank coals contain higher percentages of functional groups containing oxygen, such as hydroxyls, carbonyls, and ethers, compared to high rank coals. With increasing rank,

progressively fewer functional groups are present, the oxygen content is reduced, and the ring system becomes more polycondensed (Dorrestijn *et al.*, 2000).

NMR spectra of lignites have indicated structures that are strongly related to lignin (Schmeirs and Köpsel, 1997). Like coal, lignin is a heterogeneous, optically inactive polymer. As the major aromatic component of plant materials (crucial for the structural integrity of the plant cell wall), it is the most abundant form of aromatic carbon in the biosphere (Hofrichter, 2002; Leonowicz *et al.*, 1999; Boerjan *et al.*, 2003). Lignin is mainly derived from *p*-coumaryl, coniferyl and sinapyl alcohols that undergo dehydrogenative polymerisation via radical coupling reactions involving peroxidase, laccases, polyphenol oxidases, and coniferyl alcohol oxidase (Boerjan *et al.*, 2003). Lignin is preserved during the early coalification process, and hence, low ranking lignites show the greatest resemblance to lignin (Figure 1.3) (Gokcay *et al.*, 2001). As buried wood is gradually transformed to lignite, the lignin structural units are modified to catechol-like structures, and guaiacyl and syringyl groups are transformed to yield the aromatic structures typically found in brown coals (Table 1.1). The presence of phenols, cresols, guaiacols, catechols, eugenols, and small amounts of vanillin in brown coal were confirmed by pyrolysis gas chromatography-mass spectroscopy (GC-MS) (Hatcher *et al.*, 1988; Hatcher, 1989). Humic acids, formed during transformation of biomolecules (such as lignin) from decaying plant material and microbial activity, also make up a substantial part of the organic carbon incorporated in low rank coals (Steffen *et al.*, 2002). Structures similar to those of plant tannins and flavonoids may also be apparent (Faison, 1993).

The chemical structure of coal is complex and is still under discussion, and only representative partial structures can be found in literature (Klein *et al.*, 1988; Fakoussa and Hofrichter, 1999) (Figure 1.3). A two-phase model describing the general chemical structure of lignite has been proposed: i) a macromolecular phase represents a space network of macromolecules, ii) smaller molecules, excluded from the space network, but trapped in the network, depending on polarity and molecular geometry or linked to the network via covalent bonds, are referred to as the molecular (mobile) phase (Schmeirs and Köpsel, 1997). Younger coals such as lignite show a larger proportion of the macromolecular components than older coals. Thus, varied structural conditions exist for potential microbial depolymerisation of the macromolecular structure of lignites (Schmeirs and Köpsel, 1997).

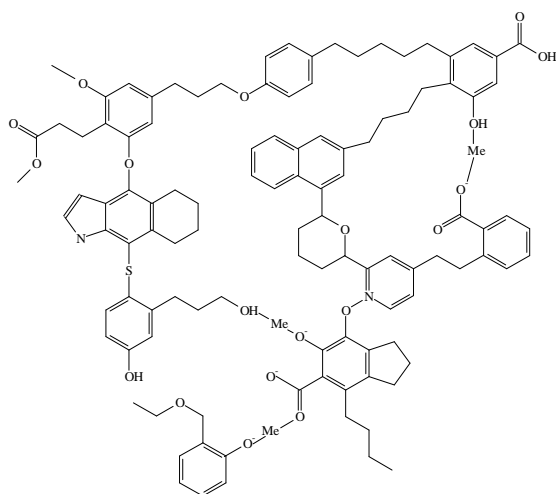
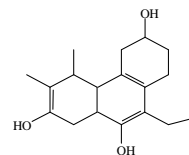
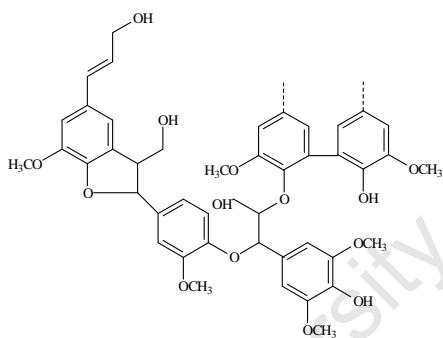
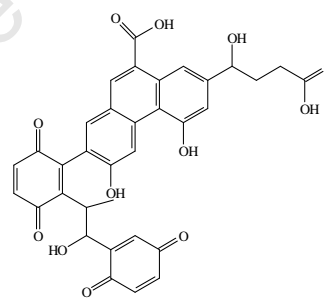











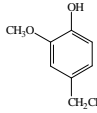
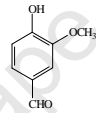
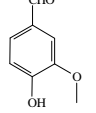

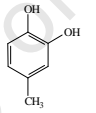

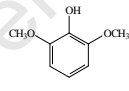
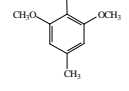
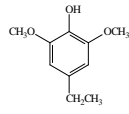
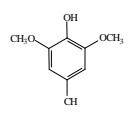
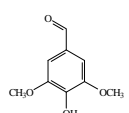
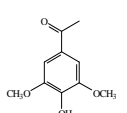
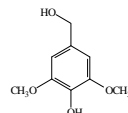

**LIGNITE *****SUB-BITUMINOUS COAL****LIGNIN****HUMIC ACID**

Figure 1.3 Comparison of model structures of low-rank coals with those of lignin and humic acid (* “Me” refers to metal ions) (Faison, 1993; Fakoussa and Hofrichter, 1999; Schulten and Schnitzer, 1993)

Table 1.1 Compounds identified in brown coals by pyrolysis-gas chromatography-mass spectrometry (adapted from Hatcher *et al.*, 1988)

Classification	Compound
Phenols and methylphenols	 phenol  o-cresol  m-cresol  p-cresol  2,4-dimethylphenol  2,3-dimethylphenol  ethylphenol
Guaiacyl phenols	 guaiacol  methylguaiacol  ethylguaiacol  vinylguaiacol  eugenol / cis-isoeugenol / trans-isoeugenol  vanillin  acetoguaiacone
Catechols	 catechol  methylcatechol  methoxycatechol
Syringyl phenols	 2,6-dimethoxyphenol  4-dimethyl-2,6-dimethoxyphenol  4-ethyl-2,6-dimethoxyphenol  4-vinyl-2,6-dimethoxyphenol  syringaldehyde  acetosyringone  syringyl alcohol
Carbohydrates	 levoglucosane

1.2.4 Reactivity of coal towards microbial degradation

Coal is a complex, insoluble solid which would appear to be resistant to microbial degradation under natural circumstances (Klein *et al.*, 1988; Hofrichter and Fakoussa, 2001). The mobile phase of coal consists of low molecular mass compounds which are more water soluble than the macromolecular phase and, therefore, are more readily available to microorganisms (Faison, 1991). Thus, many bacteria and fungi have been shown to grow on these low molecular mass compounds which are often leached into aqueous environments (Klein *et al.*, 1988; Ralph and Catcheside, 1997).

The chemical bonds in the macromolecular network of coal are very strong, with aliphatic bridges having the lowest bond energy of 218 KJ/mole. High temperatures are therefore needed to break the bonds in coal (Hodek, 1994). However, degradation of such bonds may also be achieved by ionic mechanisms at low temperatures, where solutions of alkali metals are used to saturate coal anions through protonation or alkylation reactions. It is therefore likely that coal may be accessible to microbial enzymes which can function in a similar manner to the ionic mechanisms at moderate temperatures (Klein *et al.*, 1988). Extensive research has shown that degradation of coal by fungi and bacteria can be achieved by optimising growth conditions such as humidity, mineral content, presence of additional carbon source, and by weathering the coal substrate itself (Hofrichter and Fakoussa, 2001). Exposure of coal to light, air, changing temperature and water may affect the oxidation state, moisture content, and physical integrity, making it more suitable for microbial growth (Faison, 1993). Knowledge of the proportions of the macromolecular components, their building principles, and their behaviour towards enzymatic attack is of great importance in elucidating the mechanisms of microbial biosolubilisation and depolymerisation (Schmeirs and Köpsel, 1997).

The heterogeneity and solid nature of coal impose certain limitations on its biological degradation. Firstly, the solid nature of coal demands an extracellular mechanism for breakdown, and secondly, the chemically heterogeneous substrates require unspecific enzymes or an array of enzymes that together will be able to act on the various substructures in coal (Catcheside and Ralph, 1999; Klein *et al.*, 1988). The heterogeneity and uncertainty regarding coal structure makes analysis of catabolic products and choosing an appropriate model difficult (Ralph and Catcheside, 1997). Experimental results cannot easily be transferred from one coal to another kind of coal (Klein *et al.*, 1988).

Low rank coals seem to be more porous than higher rank coals and thus allow access to larger internal surfaces, which would be necessary for more extensive degradation to occur (Faison, 1993). This is advantageous for filamentous microbes such as fungi and actinomycetes, since they can colonise insoluble substrates by mechanical penetration. The hardness of some coals would prohibit this kind of activity in microorganisms, but low rank coals are softer and less dense, contributing to the susceptibility of lignites to microbial invasion and degradation (Faison, 1993). Further, low rank coals have lower

degrees of ring condensation than high rank coals, as well as greater ring substitution with oxygen-containing functionalities, making them more amenable to biodegradation (Faison, 1993).

1.3 BIOCONVERSION OF LOW RANK COAL

Coal has not generally been regarded as a suitable substrate for microbial modification, due to the complexity of its structure and the extreme conditions involved in its genesis (Hofrichter and Fakoussa, 2001). However, coal is not sterile and indigenous microbial populations in coal were described as early as 1928 (Hofrichter and Fakoussa, 2001). Thus, a number of different microbial strains have been isolated directly from coal (Faison, 1991). In fact, the earliest publications on the action of microorganisms in coal were in 1908 (Catcheside and Ralph, 1999; Hofrichter and Fakoussa, 2001). After these first publications on microbiological action on coal, interest in this field waned until the investigations of the microbial decomposition of untreated, hard coals were reported as part of a doctoral thesis in 1981, and the ability of certain bacteria to utilize the organic fraction of hard coal as the sole carbon source and to solubilise native coal was demonstrated (Fakoussa, 1981).

Around the same time, Cohen and Gabriele (1982) reported fungal growth directly on lignite coal or in minimal medium supplemented with crushed coal. In their experiments, using *Trametes* (*Polyporus* or *Coriolus*) *versicolor* and *Poria monticola* (both wood-decaying fungi) as biocatalysts and lignite coal as the primary substrate, growth of both fungi was observed by the extension of hyphae. The solid particles were degraded to a black liquid product. No growth was observed in minimal medium without coal and the black liquid did not appear unless fungi and coal were present together. An extracellular process of coal biosolubilisation was implicated in an experiment in which coal was solubilised even though coal and fungus were separated by a membrane barrier with 0.45 µm pores.

This sparked a widespread interest in the possibility of developing biological processes to transform low rank coal to value added products (Ralph and Catcheside, 1997). To date a substantial number of studies have demonstrated that certain fungal genera and bacteria have the capacity to solubilise lignites and sub-bituminous coals (Temp *et al.*, 1999) (Table 1.2). Active fungi include *Phanerochaete chrysosporium*, *Trametes versicolor*, *Nematoloma frowardii* (subsequently reclassified as *Phlebia* sp. Nfb19), *Poria monticola*, *Bjerkandera adusta*, *Aspergillus* sp., *Fusarium oxosporium*, *Trichoderma atroviridae*, and *Pleurotus ostreatus* (Pyne *et al.*, 1987; Fakoussa and Hofrichter, 1999, Temp *et al.*, 1999; Hildén *et al.*, 2008). Due to the obvious connection with the ligninocellulosic origins of coal, ligninolytic fungi and their extracellular enzyme systems have been the most commonly studied agents of degradation (Hofrichter and Fakoussa, 2001). The ability of bacteria to degrade low rank coal was first demonstrated by Fakoussa (1981), followed by reports on the ability of *Streptomyces* sp. to depolymerise lignites in pure culture and on solid media (Gokcay *et al.*, 2001). A number of studies on coal degradation, using numerous different microbial species and coal types from various parts of the globe, have since been carried out.

Achievements in research into coal bioconversion over the past 25 years are summarised in Table 1.3 below. Particular emphasis has been placed on the low rank coals since these are more susceptible to degradation than hard coals. Most efforts to date have been focused on determining key factors for optimal coal solubilisation and understanding the mechanisms involved in the process. However, the bioconversion of coal encompasses four main areas: modification of hard coal, solubilisation, depolymerisation and utilisation of LRC. Particular emphasis will be placed on the solubilisation and depolymerisation of LRC in this review.

Table 1.2 Microorganisms capable of coal biosolubilisation/depolymerisation

Microorganisms	Type	Coal Type	Reference
<i>Pseudomonas fluorescens</i>	Bacterium	Bituminous	Fakoussa, 1988
<i>Doratomyces</i> sp.	Fungus	Lignite	Laborda <i>et al.</i> , 1999
<i>Aspergillus</i> sp.	Fungus	Lignite	
<i>Trichoderma</i> sp.	Fungus	Hard coal, sub-bituminous, lignite	
<i>Penicillium</i> sp.	Fungus	Hard coal, sub-bituminous, lignite	
<i>Fusarium oxysporum</i>	Fungus	Lignite	Hölker <i>et al.</i> , 1995
<i>Coriolus hirsutus</i>	Basidiomycete	Hard coal	Bublitz <i>et al.</i> , 1994
<i>Coprinus sclerotigenis</i>	Basidiomycete	Hard coal	
<i>Trichoderma</i> sp.	Hyphomycete	Asphaltene	
<i>Agrocybe semiorbicularis</i>	Basidiomycete	Asphaltene	
<i>Phanerochaete chrysosporium</i>	Basidiomycete	Lignite	Gokcay <i>et al.</i> , 2001
<i>Coriolus versicolor</i>	Basidiomycete	Lignite	
<i>Pleurotus sajor-caju</i>	Basidiomycete	Lignite	
<i>Pleurotus floridae</i>	Basidiomycete	Lignite	
<i>Trametes versicolor</i>	Basidiomycete	Sub-bituminous, lignite	Scott <i>et al.</i> , 1986
<i>Poria monticola</i>	Basidiomycete	Lignite	
<i>Penicillium waksmanii</i> ML20	Ascomycete	Lignite	
<i>Candida</i> sp. ML 13	Yeast	Subbituminous, lignite	
<i>Aspergillus</i> sp.	Ascomycete	Subbituminous, lignite	
<i>Sporothrix</i> sp.	Ascomycete	Subbituminous, lignite	
<i>Paecilomyces</i> sp.	Ascomycete		
<i>Pseudomonas putida</i>	Bacterium	Subbituminous, lignite	
<i>Alternaria</i> sp. F5	Deuteromycete	Lignite	Hofrichter <i>et al.</i> , 1997
<i>Phoma</i> sp.	Ascomycete	Lignite	
<i>Penicillium griseopurpureum</i>	Ascomycete	Lignite	
Aphylllophorales/Agaricales	Basidiomycete	Coal-derived humic substances	Willmann & Fakoussa, 1997
<i>Coriolus versicolor</i>	Basidiomycete	Lignite	Catcheside & Mallet, 1991
<i>Phanerochaete chrysosporium</i>	Basidiomycete	Lignite	Ralph and Catcheside, 1996
<i>Ganoderma applanatum</i>	Basidiomycete	Lignite	
<i>Perenniporia tephropora</i>	Basidiomycete	Lignite	
<i>Pleurotus ostreatus</i>	Basidiomycete	Lignite	
<i>Pycnoporus cinnabarinus</i>	Basidiomycete	Lignite	

Table 1.2 (continued)

Microorganisms	Type	Coal Type	Reference
<i>Rigidoporus ulmarius</i> <i>Xylaria hypoxylon</i> <i>Gleophyllum trabeum</i> <i>Lentinus lepideus</i> <i>Merulius tremellosus</i>		lignite lignite	Catcheside & Mallette, 1991 Ralph <i>et al.</i> , 1996
<i>Acinetobacter</i> sp. 10 <i>Pseudomonas</i> sp. 2	Bacterium Bacterium	lignite lignite	Golovin <i>et al.</i> , 1996
<i>Penicillium decumbens</i> P6	Bacteria	Lignite	Yuan <i>et al.</i> , 2006
<i>Coprinus sclerotigenis</i> <i>Agrocybe semiorbicularis</i> <i>Panus tigrinus</i> H1 <i>Mycena tintinnabulum</i> M25-1 <i>Lentinus edodes</i> A20-2	Litter-decay fungus Litter-decay fungus White-rot fungus Litter-decay fungus White-rot fungus	Hard coal Hard coal Hard coal Hard coal Hard coal	Hofrichter <i>et al.</i> , 1997
<i>Nematoloma frowardii</i> b19 * <i>Clitocybula duseni</i> b11	Basidiomycete Basidiomycete	Lignite Lignite	Hofrichter & Fritsche, 1997 Hofrichter <i>et al.</i> , 1999
<i>Phanerochaete chrysosporium</i>	Basidiomycete	Brown coal	Ralph & Catcheside, 1994, 1999
<i>Bacillus</i> sp	Bacterium	Lignite	Crawford & Gupta, 1991
<i>Pycnoporus cinnabarinus</i> <i>Polyporus ciliatus</i>	Basidiomycete Basidiomycete	Coal humic acids	Temp <i>et al.</i> , 1999
<i>Poria vaporaria</i> <i>Pholiota aurivella</i> <i>Piptoporus betulinus</i> <i>Fusarium culmorum</i> <i>Marasmius scorodoni</i> <i>Laetiporus sulphureus</i>	Basidiomycete Basidiomycete Basidiomycete Deuteromycete Basidiomycete Basidiomycete	Organic coal extracts	Osipowicz <i>et al.</i> , 1996
<i>Polyporus versicolor</i> <i>Poria monticola</i>	Basidiomycete Basidiomycete	Lignite Lignite	Cohen & Gabriele, 1982; Pyne <i>et al.</i> , 1987, 1988; Cohen <i>et al.</i> , 1987 Fredrickson <i>et al.</i> , 1990 Fakoussa & Frost, 1999
<i>Trichoderma atroviride</i> <i>Fusarium oxysporum</i>	Deuteromycete Deuteromycete	Lignite	Monkemann <i>et al.</i> , 1997 Holker <i>et al.</i> , 1997; 1999 Hölker & Höfer, 2002 Hölker <i>et al.</i> , 2002
<i>Trichoderma atroviride</i>	Deuteromycete	Sub-bituminous coal	Silva-Stenico <i>et al.</i> , 2007 Oboirien <i>et al.</i> , 2008

* *Nematoloma frowardii* was reclassified as *Phlebia* sp. Nf19 in 2008 (Hildén *et al.* 2008)

Table 1.3 Advances in coal bioconversion (adapted from Hofrichter and Fakoussa, 2001)

Year	Achievement	Reference
1981	Effects on hard coals by <i>Pseudomonas</i> spp., simultaneous biotenside secretion	Fakoussa (1981)
1982	Solubilisation of lignite to droplets on agar plates by action of wood-decaying basidiomycetous fungi	Cohen and Gabriele (1982)
1986	Acceleration of solubilisation by pre-treatment of coal (fungi + bacteria)	Scott <i>et al.</i> (1986), Grethlein (1990) and others
1987	First solubilisation mechanism elucidated: production of alkaline substances (fungi + bacteria)	Quigley <i>et al.</i> (1987, 1988a, 1989a)
1987	Syringaldazine oxidase activity found to be active in leonardite solubilisation by <i>C. versicolor</i> (Later, involvement of syringaldazine oxidase refuted)	Pyne <i>et al.</i> (1987) Fredrickson <i>et al.</i> (1990)
1988	Second solubilisation mechanism elucidated: production of chelating agents (fungi)	Cohen <i>et al.</i> (1990), Quigley <i>et al.</i> (1988b, 1989b)
1989	First product on the market: solubilised lignite as fertilizer	Arctech, Virginia (USA)
1990	Coal biosolubilisation by thermophilic microorganisms	Runnion and Combie (1990)
1990	Kinetic studies on biosolubilisation of lignite: importance of oxygen partial pressure in biosolubilisation process (bacteria and fungi) might influence choice of bioreactor.	Moolick <i>et al.</i> (1990)
1990	Preliminary studies on anaerobic conversion of microbially solubilised coal (Microbial conversions of coals to clean fuels – production of methane: precursors = methanol, ethanol, propanol, butanol, acetic acid, propionic acid, butyric acid and valeric acid)	Davidson <i>et al.</i> (1990) Barik <i>et al.</i> (1988)
1990	First isolation and identification of coal solubilising product, ammonium oxalate monohydrate, produced by <i>T. versicolor</i> (basidiomycetous fungus)	Cohen <i>et al.</i> , (1990) Torzilli and Isbister (1994)
1991	Evidence that chelators alone are not responsible for all effects	Fakoussa and Willmann (1991), Fakoussa (1994)
1994	Decolourisation and reduction of MW of soluble lignite derived humic acids proves catalytic, i.e. enzymatic attack (basidiomycetous fungi)	Willmann (1994), Ralph and Catcheside (1994), Hofrichter and Fritsche (1996, 1997a)
1991	Improved analysis by ¹³ C-solid state NMR, MW determination, e.g. ultrafiltration, gel permeation chromatography	Willmann and Fakoussa (1991), Polman and Quigley (1991), Ralph and Catcheside (1996), Hofrichter and Fritsche (1996, 1997a), Henning <i>et al.</i> (1997) and others
1995	Solubilisation of low rank coal controlled by type of carbon source: cultivation on glutamate or gluconate induced coal-solubilising state (deuteromycetous fungi)	Hölker <i>et al.</i> (1995)
1996	In-vitro systems shown to preferentially polymerise humic acids without regulation of the fungus (laccase)	Willmann (1994), Frost (1996) and others
1997	In-vitro systems based on fungal Mn peroxidase shown to depolymerise humic acids and to attack coal particles, including the matrix	Hofrichter and Fritche (1997b), Hofrichter <i>et al.</i> (1999), Mönkemann <i>et al.</i> (1997)

Table 1.2 (Continued)

Year	Achievement	Reference
1997	Involvement of unspecific esterases and ligninolytic enzymes in the solubilisation of coal (deuteromycetous fungi)	Hölker <i>et al.</i> (1997, 1999), Mönkemann <i>et al.</i> (1997), Laborda <i>et al.</i> (1997, 1999)
1997	First fine chemical successfully produced from heterogenous humic acid mixtures by bacterial pure cultures: polyhydroxyalkanoates (PHA, 'Bioplastics')	Steinbüchel and Fächtenbusch (1997), Fächtenbusch and Steinbüchel (1999)
1999	Involvement of laccase in depolymerisation of coal implied by conversion of coal humic acids to fulvic acids <i>in vivo</i> by <i>T. versicolor</i> (basidiomycetous fungus)	Fakoussa and Frost (1999)
1999	Involvement of hydrolytic and oxidative enzymes in solubilisation of low rank coal by <i>T. atroviride</i> (deuteromycetous fungus), also tested by using ¹⁴ C-labelled lignite	Hölker <i>et al.</i> (1999, 2002)
2001	2 level 3 variable factorial design incorporating solids loading, acetate as C-source and particle size of coal. Preliminary gasification tests with solubilised coal yielding 21% energy recovery through gaseous methane product.	Gokcay <i>et al.</i> (2001)
2002	9.3% solubilisation of lignite by solid state fermentation with <i>T. atroviride</i> in a new trickle-bed reactor	Hölker and Höfer (2002)
2006	Mechanisms of coal bisolubilisation in <i>P. decumbens</i> P6 combination of production of alkaline materials, peroxidase and esterase. First report on involvement of biosurfactant in coal solubilisation by fungi.	Yaun <i>et al.</i> (2006)
2007	Degradation of low rank coal by <i>T. atroviride</i> (ES 11) produced 4-hydroxyphenylethanol, 1,2-benzendiol, and 2-octenoic acid. Presence of intracellular aromatic, ring cleavage enzymes detected. Intimate association between coal particles and fungal mycelia facilitates transfer of depolymerisation products into cell.	Silva-Stenico <i>et al.</i> (2007)
2007	Key operating variables that influence coal biosolubilisation in a slurry bioreactor identified: coal loading and particle size (deuteromycetous fungus)	Oboirien <i>et al.</i> (2008)

1.3.1 Biosolubilisation of LRC

The biosolubilisation of coal, leading to the formation of a liquid product containing solubilised coal macromolecules, occurs as a result of the microbial production of alkaline substances and/or chelating agents and surfactants. It is, therefore, a non-enzymatic dissolving process that usually occurs at alkaline pH (7-10), and does not necessarily result in a decrease in the average molecular mass of coal macromolecules (depolymerisation) (Hofrichter and Fakoussa, 2001). The use of surface cultures, to which sterile coal particles are added, has been a commonly used method to test a microorganism's ability to biosolubilise coal. The appearance of liquid droplets on the surface of coal was taken as an indication of a biosolubilisation reaction (Scott *et al.*, 1986; Cohen and Gabriele, 1982; Catcheside and Mallet, 1991; Gokcay *et al.*, 2001; Başaran *et al.*, 2003). Diffusion of dark liquids in the medium occurred in some

instances where the extent of coal biosolubilisation was high (Cohen and Gabriele, 1982; Catcheside and Mallet, 1991).

Scott *et al.* (1986) conducted an investigation in which microbial solubilisation of coal was studied using both surface and submerged cultures of various fungi. The liquid product has varied from clear water to black, and in the most successful tests, the coal was almost totally solubilised after 7-14 days. Of the fungi used in this experiment (*Trametes versicolor*, *Poria monticola*, *Penicillium waksmanii*, *Candida* sp., *Aspergillus* sp., *Sporothrix* sp. and *Paecilomyces* sp.), *Candida* sp. was most effective, showing an average degree of biosolubilisation of 87%. Attachment of fungal mycelia to coal particles was observed when the same fungi were grown in suspension cultures (submerged cultures). Notable colouration of the supernatant occurred, which was not observed in the absence of the fungi. Thus, it was concluded that direct contact between the organisms and the coal surface increased the rate of biosolubilisation. The elemental analysis of the liquid product, compared with that of the native coal indicated similar carbon content, decreased hydrogen and oxygen content, increased soluble sulphur content and increased nitrogen content. The increase in nitrogen may have been an indication of the formation of active metabolites needed to solubilise the coal. The lyophilized aqueous liquid product was soluble in water, which indicated that the organic material in the product was very water-soluble. Gel permeation chromatography of the liquid product indicated that the major portion of the components had a molecular weight between 30 and 300 kDa. However, the solubility of the bulk of the organic material in water was also pH-dependent; this pH-dependent solubility, coupled with the high molecular weight of the material, was consistent with production of organic compounds such as humic acids. An increase in solubility with successively increasing polar solvents suggested that the material was highly polar. Nuclear magnetic resonance (NMR) and infrared (IR) analysis suggested that the product was highly aromatic in nature, possibly containing aromatic hydroxyl (phenolic) groups. The presence of primary amines and carbonyl groups was also suggested. HPLC analysis indicated the presence of a number of ultraviolet-absorbing and fluorescent species which were not identified. Protein analysis by SDS-PAGE indicated the presence of a protein of molecular weight approximately 60 kDa; this was most likely to be an extracellular enzyme that contributed to the solubilisation process (Scott *et al.*, 1986).

Similar results to those described above have been reported by various authors who have investigated biosolubilisation of lignite. Typically, the chemical characterisation of biosolubilised coal obtained from shake flask cultures was limited to qualitative analysis, with solubilisation commonly measured spectrophotometrically as an increase in absorbance at 400-450 nm (Wilson *et al.*, 1987; Cohen *et al.*, 1987; Shin *et al.*, 1995; Gokcay *et al.*, 2001). Quantitative gravimetric analysis of residual coal, recovered after bioconversion with fungi, was problematic due to the attachment of mycelia to coal particles (Catcheside and Mallet, 1991; Fakoussa, 1988). Generally, the rate and degree of coal biosolubilisation appeared to be affected by the type of microorganism; the rank, oxidation state and particle size of coal;

and the pH of the supernatant (Scott *et al.*, 1986; Machinikoskwa, 2002; Quigley *et al.*, 1989; Gokcay *et al.*, 2001; Catcheside and Mallet, 1991 and Cohen *et al.*, 1987).

Several authors have reported that coal solubilisation may be non-enzymatic and may result from the production of alkaline materials by microorganisms. Collectively, research to date points to at least three possible mechanisms of coal biosolubilisation (each discussed below):

- The production of alkaline substances
- Presence of certain chelators
- Action of esterases (Fakoussa and Frost, 1999)

1.3.1.1 Production of alkaline substances

Reports on experiments concerning the influence of pH on coal biosolubilisation suggest that the production of alkaline substances could be involved in the process. Cohen *et al.* (1987) showed that the rate of coal biosolubilisation increased dramatically as the pH of the solution was increased from pH 6. In a comparison made by Quigley *et al.* (1988) between biosolubilised coal and alkali-extracted coal, both oxidised with nitric acid prior to solubilisation, similar results were obtained from UV-visible spectral scans, suggesting that alkali production is a mechanism by which microorganisms solubilised oxidised coals (Quigley *et al.*, 1988).

Later, further evidence of the involvement of microbially produced alkaline substances in coal biosolubilisation was presented by Quigley *et al.* in 1989, where experiments involved the use of plate assays *i.e.* the addition of coal particles to culture lawns on agar plates. Biosolubilisation was visible by the appearance of liquid droplets on the surface, which were harvested. The final pH of the agar medium was measured, and the results suggested that the increase in pH was dependent on the nitrogen concentration. The source of alkalinity was attributed to presence of ammonia, polyamines or polypeptides. An increase in the oxygen content of coal was related to an increase in its carboxylic groups, which resulted in increased solubility in strong alkali. It was thus suggested that neutralisation of carboxylic acid groups in coal lead to its biosolubilisation. The mechanism proved to be independent of microbial species or type of coal and a direct relationship between coal alkali solubility and coal biosolubility suggested a common solubilisation mechanism (Quigley *et al.*, 1989).

1.3.1.2 Presence of certain chelators

Metal ions, which form salt bridges linking organic moieties in coal, are important elements in the structure of low-rank coals and hence, the biosolubilisation of coal may be due to the action of microbially-produced chelators which sequester the metal ions (Fredrickson *et al.*, 1990; Pyne *et al.*, 1987). It has been shown that the addition of citrate (at concentrations between 0.1 and 0.3 mM) stimulated the biosolubilisation of leonardite by an extracellular fraction from *T. versicolor*, which also contained syringaldazine oxidase activity. At concentrations greater than 0.3 mM, citrate effectively solubilised leonardite, but inhibited the oxidase activity at concentrations greater than 1mM. At low citrate concentrations, chelation and the action of the oxidase enzyme were additive, but at higher citrate concentrations, direct inhibition of the net biosolubilising activity was observed, suggesting that biosolubilisation was not caused by chelation alone (Pyne *et al.*, 1987).

Quigley *et al.* (1989) also investigated the effects of multivalent cations on low rank coal solubility in alkaline solutions and microbial cultures. Coal solubilities were measured in the presence of certain chelators such as EDTA, nitrilotriacetic acid and citrate, and the coal was found to be more soluble when the known chelators were added. The addition of multivalent cations caused precipitation of the coal and a decrease in pH. The authors therefore deduced that the primary cause of enhanced coal solubility in alkali was the removal of organically bound metal ions, and that the ability of microorganisms to acquire metal ions from the growth medium or coal effected biosolubilisation. The primary means of obtaining metal ions by microorganisms is the production of extracellular metal chelators, able to solubilise insoluble metal ions, thereby making them available to the microbes (Quigley *et al.*, 1989). Coal biosolubilisation therefore also occurs as a result of the sequestration of metal ions from coal by microorganisms.

Cohen *et al.* (1990) described the isolation and chemical identification of the first biologically produced compound that solubilises low-rank coal from *T. versicolor*. The coal-solubilising agent (CSA) was separated from the broth in which *T. versicolor* was grown, using reverse-phase HPLC and size exclusion chromatography, ethanol fractionation and recrystallization. The infrared spectrum (IR) of the isolated CSA and ammonium oxalate monohydrate were identical, and the chemistry exhibited by the CSA, its NMR and IR spectra, and its crystal structure (as the monohydrated ammonium salt) confirmed that the active agent in the dissolution of low-rank coals by *T. versicolor* was the oxalate anion. However, in similar experiments carried out by Torzilli and Isbister (1994) using *T. versicolor* to biosolubilise lignite, the effect of oxalate could only account for some, but not all of the coal biosolubilisation observed. The production of chelators may therefore be only one of the mechanisms used by *T. versicolor* to solubilise lignite, and this may not necessarily be effective in biosolubilising other coals.

1.3.1.3 Action of esterases

Crawford and Gupta (1991) described the depolymerisation of soluble coal polymers by bacteria which were isolated from soil associated with a coal seam. A decrease in the principal polymer peak from approximately 174 kDa to 113 kDa was observed. Elemental analysis of the depolymerised coal, compared with the starting coal, showed no major changes in the carbon and oxygen content, suggesting that a non-oxidative enzymatic process had occurred. The authors speculated that the possible cause of depolymerisation was due to the cleavage of ether and ester bonds by esterases produced by the bacteria (Crawford and Gupta, 1991). There are considerable numbers of ester linkages in low rank coal but the involvement of esterases in coal solubilisation is questionable because esterases are not known to use mediator molecules, and further, esterases cannot enter the tight macromolecular structure of coal due to steric hindrances (Große, 2000; Hofrichter and Fakoussa, 2001). Although there have been other reports on the involvement of esterases from fungi in the biosolubilisation of coal, only a small portion of ester linkages is cleaved and more research is required to confirm the role of esterases in coal biosolubilisation (Campbell *et al.*, 1988; Cohen *et al.*, 1990; Hölker *et al.*, 1997, 1999; Laborda *et al.*, 1997, 1999; Große, 2000; Hofrichter and Fakoussa, 2001).

1.3.2 Depolymerisation of LRC

The depolymerisation of coal is an enzymatic process which causes cleavage of C-C or C-O linkages, resulting in the lowering of the average molecular mass of coal macromolecules (Hofrichter and Fakoussa, 2001). Thus, depolymerisation of large coal macromolecules is an essential pre-requisite for the production of low molecular products from the bioconversion of LRC (Ralph and Catcheside, 1997). This biodepolymerisation offers an attractive alternative to thermo-catalytic conversion, avoids the production of toxic byproducts, and also results in a product that would be more readily transformed into value-added chemicals than native low-rank coal (Ralph and Catcheside, 1997).

To effectively demonstrate that the macromolecular fraction of low rank coal has been depolymerised by biological agents: i) it must be recovered after exposure to the agent and its average molecular mass must show a decrease, ii) coal monomers must be produced *de novo*, or iii) products such as CO₂, CH₄ or cell biomass must be derived from coal carbon (Ralph and Catcheside, 1997). In most reported studies on coal bioconversion, biosolubilisation has not been accompanied by depolymerisation. However, Pyne *et al.* (1987) reported that cell free extracts of *Trametes versicolor* could solubilise leonardite (weathered lignite) to form products with lower average molecular masses. In another experiment by Ralph and Catcheside (1997), numerous solvent-extractable low-molecular mass compounds were detected by gel permeation chromatography, when lignite was solubilised by *P. chrysosporium* and *T. versicolor*. However, whether these compounds were produced from coal macromolecules *de novo*, or whether they constituted the coal mobile phase, released upon solubilisation, was unclear.

Hofrichter and Fritche (1996) developed a screening system to select fungi with a high potential for depolymerisation of low-rank coals, where the ability to depolymerise coal was related to the ability of the fungi to decolourise agar media containing coal-derived humic acids (prepared from lignite by extraction with sodium hydroxide solution, also known as alkali-solubilised coal). The high molecular-mass fractions of coal humic acids were depolymerised to form fulvic acid-like compounds. The ability to decolorize the dark brown agar and form yellowish products was found to be limited to white-rot fungi belonging to the Basidiomycetes. Bleaching effects occurred around the growth area of the fungi, but coal humic acids cannot be taken up into the hyphae of the fungus due to their size, and thus, an extracellular enzymatic attack was assumed. Oxidase and peroxidase enzyme activities were detected in the decolourised growth zone of the fungi by extracting the agar samples with a buffer and measuring the oxidation of the substrate, 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS).

1.3.3 Role of ligninolytic enzymes in the depolymerisation of coal

The evidence pertaining to coal bioconversion has generally indicated an extracellular enzymatic process, and the infrared spectrum of the water-soluble products from coal bioconversion indicated the presence of carboxyl and hydroxyl groups, which is consistent with an oxidative degradation process (Fakoussa, 1988). The ability of microorganisms to depolymerise coal macromolecules has also been related to the production of extracellular oxidative enzymes (Scott *et al.*, 1986; Hofrichter and Fritche, 1996; 1997a, 1997b; Willman and Fakoussa, 1997). These enzymes, mainly produced by white-rot fungi, are also involved in the degradation of lignin, and are mainly produced during growth-limiting (stress) conditions *i.e.* during stationary phase of the fungal growth cycle. The ligninolytic enzyme system consists primarily of peroxidases *i.e.* lignin peroxidases, manganese peroxidases, and phenol oxidases or laccases (Tien and Kirk, 1983; Kirk and Farrell, 1987; Field *et al.*, 1993, Vyas *et al.*, 1994). These enzymes belong to a large group of enzymes known as the oxidoreductases that catalyse biological oxidation/reduction reactions (May 1999).

Peroxidases have the ability to convert phenolic compounds by polymerization and precipitation processes, due to the oxidation of aromatic compounds using hydrogen peroxide to oxidise the enzyme into a catalytically active form (Zhang and Nicell, 1999). When peroxidases are incubated with H₂O₂ and coal in either aqueous or non-aqueous solutions, coal solubilities increase; the increases have been attributed to the action of peroxidases, although H₂O₂ itself is reactive with coal. Therefore, it is not known whether the rate of the enzyme-catalyzed reaction is higher than the non-enzymatic chemical reaction (Quigley *et al.*, 1991).

Lignin peroxidase (LiP) was first isolated from *Phanerochaete chrysosporium* (Tien and Kirk, 1983). It is a glycoprotein that contains iron protoporphyrin IX (heme) as a prosthetic group. It has a broad substrate specificity for aromatic compounds, oxidising both phenolic and non-phenol structures, and is unique in its ability to oxidise methoxylated, non-phenolic aromatic compounds of high redox potential (Fakoussa and Hofrichter, 1999).

LiP is oxidised by H_2O_2 to a form which has two oxidising equivalents. Compound I is converted to a second state, compound II, and then to its resting state via two consecutive, single electron reductions (Ralph and Catcheside, 1997) (Figure 1.4A). Compound I can abstract an electron from non-phenolic aromatic compounds to yield an aryl cation radical which in turn can undergo a variety of non-enzymatic reactions such as C-O and C-C fission and ring cleavage. The radicals can act as diffusible oxidants once they have reacted with O_2 and H_2O , and can disproportionate or polymerise. LiP is also relatively sensitive to inactivation by its own co-substrate, H_2O_2 , or phenolic substances. Nevertheless, isolated LiP has the potential to degrade high molecular mass coal substances. Wondrack *et al.* (1989) first demonstrated the oxidizing effect of lignin peroxidases from *P. chrysosporium* on coal polymers in aqueous solution. LiP caused the disappearance of high molecular mass coal fractions and the formation of smaller ones. The depolymerizing activity was stimulated by the addition of veratryl alcohol, a mediator in the reaction (Hofrichter and Fritche, 1996). LiP may catalyse the one electron oxidation of veratryl alcohol to form a cation radical which is able to diffuse from the active site and oxidise bulky substrates, such as coal (Zapanta and Tien, 1997). Veratryl alcohol is also known to protect LiP from inactivation by H_2O_2 (Wondrack, 1989), and can be used to measure LiP activity by monitoring its conversion to form veratryl aldehyde (Kirk *et al.* 1986).

In contrast, equivalent degrees of biotransformation of alkali-solubilised low rank coal by *P. chrysosporium* in the presence and absence of metavanadate, an inhibitor of LiP, implied that LiP activity was not essential for extracellular catabolism of coal molecules. LiP may have catalysed cleavage of low molecular weight structures from the surface of coal macromolecules, leaving resistant cores with reduced absorbance, but with little change to the relative apparent molecular mass of the alkali-solubilised coal as measured by gel permeation chromatography. However, depolymerisation may have been undetected due to an increase in the hydrodynamic volume of alkali-solubilised coal, caused by solvation (Ralph and Catcheside, 1997). Since the methoxy content of low rank coal is lower than that of lignin, generation of aryl cation radicals by LiP is anticipated to play a more important role in lignin degradation than in the degradation of low rank coal (Ralph and Catcheside, 1997).

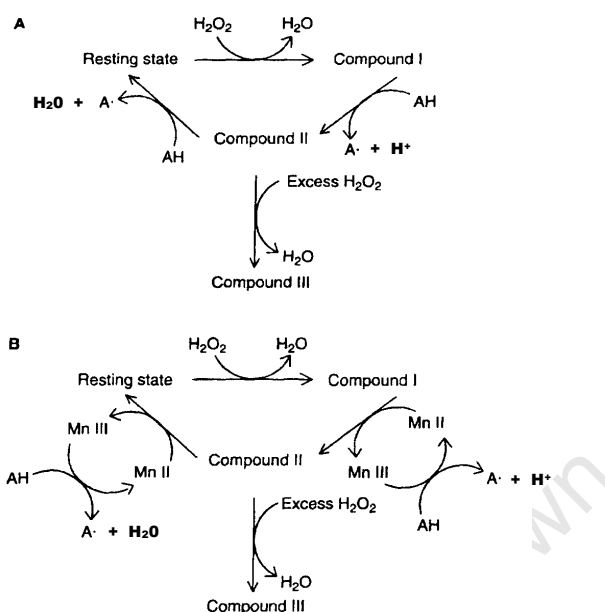


Figure 1.4 Catalytic cycles for lignin peroxidase (A) and manganese peroxidase (B) (Gold and Alic, 1993)

Manganese peroxidase (MnP) resembles LiP in that it is glycosylated and contains heme as its prosthetic group. However, MnP compounds I and II oxidise Mn(II) to Mn(III) which in turn form stable, diffusible complexes with α -hydroxy organic acids, such as lactate, malonate, and oxalate, that can then oxidise phenolic compounds to phenoxy radicals. These radical centres can rearrange resulting in C-C fission (Ralph and Catcheside, 1997) (Figure 1.4B). Suggestions that native low-rank coal and the coal matrix are accessible to attack by MnP have been substantiated by recent research (Fakoussa and Hofrichter, 1999). Low rank coal has a higher content of phenolic hydroxyl groups than lignin, and thus, is likely to be highly reactive towards MnP (Ralph and Catcheside, 1997). Incubation of MnP with coal particles resulted in the formation of small humic acid and fulvic acid fragments (Fakoussa and Hofrichter, 1999), and characteristic changes in the surface morphology of coal *i.e.* formation of crater-like structures, resulted from direct attack by MnP. MnP's action via the redox mediator couple, Mn(II)/Mn(III), generated during depolymerisation, and its small size that enables diffusion into parts of the coal, was suggested to give it an advantage over larger enzymes (Fakoussa and Hofrichter, 1999).

MnP action on coal has been shown to be dependent on oxygen tension. Thus, MnP from *P. chrysosporium* polymerised alkali-solubilised low rank coal under ambient oxygen tension and depolymerised it under hyperbaric oxygen without altering the absorbance of the coal (Ralph and Catcheside, 1997). It was suggested that MnP and LiP might operate synergistically to convert low rank coal to products that may be further metabolised intracellularly by *P. chrysosporium*.

Nevertheless, however promising the ligninolytic peroxidases may be for coal bioconversion, they may not be favourable in terms of commercial applications due their requirement of the relatively expensive cofactor, hydrogen peroxide.

A notable number of fungi are known to produce the enzyme laccase, including *Trametes versicolor*, *Neurospora crassa* and *Pleurotus ostreatus*. The laccase molecule is a dimeric or tetrameric glycoprotein containing four copper atoms per monomer (Gianfreda *et al.*, 1999). The catalytic cycle of laccase comprises several one-electron transfers between the copper atoms while oxygen is bound to the active site of the enzyme (Figure 1.5). Laccase catalyzes the oxidation of various aromatic compounds, specifically phenols and aromatic amines, while concomitantly reducing molecular oxygen to water. Thus, laccase is capable of oxidizing both phenolic and non-phenolic compounds. The action of laccase on phenolic aromatics results in the formation of phenoxy radicals, which readily undergo non-enzymic reactions such as radical-radical coupling, disproportionation, deprotonation and nucleophilic attack by water, leading finally to depolymerization (Fakoussa and Hofrichter, 1999). Due to the low redox potential of laccase, it is unable to oxidise non-phenolic substances in the absence of primary laccase substrates acting as mediators e.g. ABTS. These mediators, once oxidised by laccase, go on to oxidise non-phenolic substances of higher reducing potential or other larger substrates that are not accessible to the enzyme active site due to their size (Bourbonnais & Paice, 1990; Banci *et al.*, 1999).



Figure 1.5 General laccase reaction

In experiments carried out by Temp *et al.* (1999), laccase was shown to be the only ligninolytic enzyme secreted by *P. cinnabarinus* during coal humic acid degradation. This result was in accordance with previous work done by the same group that demonstrated that only laccase, and no LiP or MnP, is produced under ligninolytic conditions. The laccase production correlated with notable reduction in colour of the culture supernatant and the conversion of coal derived humic acids to fulvic acid-like compounds. This result was contested by that of Ralph *et al.* (1996), who did not observe decolourisation or depolymerisation of a soluble coal fraction by a different strain of *P. cinnabarinus*.

Furthermore, a laccase extract from *T. versicolor*, appeared to be active towards leonardite. Purification of the laccase fraction from *T. versicolor* showed that it contained two proteins: one that showed laccase activity but had little effect on coal, while the other had little laccase activity, but high coal-solubilizing activity (Cohen *et al.*, 1987; Pyne *et al.*, 1987). The latter protein was found to possess esterase activity, but subsequent experiments have shown that the esterase had much less coal biosolubilising activity than other components produced by *T. versicolor*, such as laccase and chelators (Cohen *et al.*, 1990;

Fredrickson *et al.*, 1990; Fakoussa and Frost, 1996). The decolourisation of coal-derived humic acids by *T. versicolor* was observed by Fakoussa and Frost (1996), and coincided with the production of a large amount of laccase. No peroxidase activity was detected, confirming that laccase was responsible for the decolourisation reaction. Thus, laccase has potential to bioconvert low rank coal, and its application would be economically advantageous because it only needs molecular oxygen as a cofactor.

In addition to laccase, tyrosinase (another phenol oxidase) has also been tested for its coal-biosolubilising abilities. It has been reported that a tyrosinase-like enzyme found in *N. crassa* has coal solubilising activity and that tyrosinase negative mutants of this fungus could not transform lignite particles on agar to black liquid (Fakoussa and Hofrichter, 1999).

1.4 BIOREACTOR SYSTEMS FOR COAL BIOCONVERSION BY WRF

"Bioreactors are engineered systems in which the activity of living cells is harnessed" (Ramkrishna, 2003). Bioreactors are different from conventional chemical reactors in that they are dynamic, due to changes in cellular metabolism. The development of any bioprocess is not only dependent on the selection of suitable microorganisms but also optimisation of process parameters, which include the type of bioreactor chosen (Pandey, 2003). In this section, bioreactor systems previously used in the bioconversion of coal, and bioreactor configurations suitable for cultivation of common coal degrading microbial systems are discussed.

1.4.1 Bioreactors for coal bioprocessing

Examples of coal bioprocesses include beneficiation for the removal of sulphur, conversion of coal to liquids and gaseous products, removal of hazardous components, environmental treatment of effluents and residues, and production of valuable compounds from biosolubilised/depolymerised coal. Each application requires careful consideration of optimal bioreactor configurations (Scott, 1990). Choosing appropriate bioreactor systems for coal bioprocessing becomes necessary when the process development phase begins. Reactors used in coal bioprocessing need to be simple, inexpensive to build and should perform more than one function in order to be economically viable (Andrews and Noah, 1997).

In investigations of coal biosolubilisation, packed-bed and fluidised-bed bioreactor configurations have been considered, based on surface and suspension culture experiments, respectively (Scott *et al.*, 1986). The use of packed-bed and fluidised-bed bioreactors was conceptualised by Scott *et al.* (1986) and Scott (1990), but experimental results were only presented for the fluidised-bed bioreactor system. Conventionally, the packed-bed (or fixed-bed) system would consist of a bed of stationary coal particles exposed to whole microbial cells or isolated enzymes. Initially, an inoculum and nutrient solution would be

added to the top of the column and allowed to trickle down the bed. The liquid product and residual solids would flow to the bottom and be collected whilst fresh coal would be added at the top of the column. The liquid phase flow and addition of humid air into the system would be continuous. This system can be employed when long retention times are required and coal particles are relatively large (Scott, 1990). Small coal particles would be better suited to fluidised-bed reactors, where shorter residence times are utilised (Scott, 1990). The smaller particles would be suspended in an upflowing liquid stream of reactant containing the biocatalyst. Good aeration can be maintained through a continual supply of air to the bottom of the reactor; the liquid product would be collected as a side stream, and fresh coal added to the top of the bioreactor. If additional fluidisation is required, the liquid can be recycled (Scott, 1990).

Preliminary experiments were carried out on the biosolubilisation of lignite by *Candida* sp. ML13 in a small, tapered fluidised-bed bioreactor (Scott *et al.*, 1986). The lignite was fluidised by pumping a mineral salts solution through the column, while the effluent was returned to a reservoir where it was aerated prior to being recycled. A small amount of abiotic solubilisation was evident in the control experiment, while a notable increase in soluble coal components, measured as an increase in absorbance at 292 nm, was observed in the presence of *Candida* sp. ML13. Microbial attachment occurred rapidly despite the high-hydrodynamic shear fields in the fluidised-bed bioreactor.

A small fluidised-bed bioreactor, similar to the one described above, was used in a study of the conversion of coal to a liquid product by chemically modified reductive enzymes in organic media, because this mode of operation was considered more appropriate for scale-up (Scott *et al.*, 1994). The reductive enzymes (Cytochrome c, alcohol dehydrogenase and hydrogenase) were modified with dinitrofluorobenzene, resulting in the addition of dinitrophenyl groups to the enzyme molecules. Pyridine, containing the mixture of modified enzymes, was periodically replenished and bituminous coal residue was maintained in the reactor. 43% conversion of bituminous coal to liquids, based on solids content reduction, was achieved in 24 hours.

Wilson (1987) demonstrated 8–10 % coal biosolubilisation, based on change in absorbance at 450 nm, in packed columns. The most recent bioreactor study, carried out by Hölker and Höfer (2002), involved the biosolubilisation of lignite by *Trichoderma atroviride* in a 25 litre trickle-bed bioreactor. 1.5 kg lignite and 2 litres of a pre-grown *T. atroviride* culture, containing 1.2% (w/v) glutamate as an additional carbon source, were added to the bioreactor at the start of the experiment. Following this, the cultivation was carried out in a semi-continuous, fed-batch manner where one litre of culture fluid was harvested daily and the corresponding volume replaced by distilled water. 9.3% of the lignite added to the bioreactor was solubilised but sterility was only maintained until the twentieth day of growth because the lignite used was not pre-sterilised. Thereafter a mixed culture, consisting of bacteria and metazoa developed, and by the fortieth day no *T. atroviride* was detectable. These findings showed that the solubilised lignite was not sufficient to support growth of *T. atroviride* once the additional carbon source (glutamate) was depleted,

since its growth was overtaken by the indigenous microbial population in the lignite. Although this type of trickle-bed bioreactor was suitable for lignite biosolubilisation by *T. atroviride*, the extent caused by the indigenous microbial population was unaccounted for. An investigation of this indigenous population may have been useful in identifying a new microbial strain with high coal biosolubilising activity.

More recently, the bioconversion of pre-treated bituminous hard coal in a fixed-bed perfusion column was demonstrated. Here, the fixed-bed perfusion column was used to simulate a bituminous hard coal dump environment where a symbiotic relationship between *Cynodon dactylon* (Bermuda grass), and mycorrhizal and non-mycorrhizal fungi has been observed. The fungi attack the coal, releasing humic soil-like material in the root zone, which enabled the growth of *C. dactylon* (Igbini, 2007).

1.4.2 Bioreactors for the application of WRF

Since filamentous white rot fungi (and their enzymes) are the most commonly studied microbial systems in coal degradation, it is necessary to consider bioreactors that are suitable for their cultivation. Commonly used reactors for the production of ligninolytic enzymes produced by WRF include conventional continuously stirred tank reactors (CSTRs), airlift loop reactors (ALRs), and bubble column reactors (Moreira *et al.*, 1998). These bioreactor configurations allow for homogeneous, submerged fermentations (SmF). However, solid-state fermentations (SSF) are more appropriate for filamentous fungi since this configuration closely mimics their natural habitat, and further, the enzyme activities of these fungi are typically sensitive to mechanical agitation (Pandey, 2003; Durand, 2003; Rodriguez Couto *et al.*, 2002; Fenice *et al.*, 2003; Durand, 2003). Solid-state fermentation is defined as the cultivation of microorganisms involving solids, in the absence (or near absence) of free water. The substrate must, however, possess enough moisture to support growth and metabolism of microorganisms (Pandey, 1992). SSF bioprocesses have been applied to the bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biotransformation of crops and/or crop residues for nutritional enrichments, biopulping, and production of value-added products such as antibiotics, plant growth factors, enzymes, biosurfactants, and biofuels (Pandey, 2003). Although SSF have low energy requirements and produce less wastewater, they are not without limitations (Pandey 2003). These include low mass transfer and substrate utilisation rates due to the absence of mixing. Also, agitation is recommended when oxygen availability in the mycelium is restricted.

In a study carried out by Fenice *et al.* (2003) on the bioremediation of olive mill wastewater by WRF, three reactor configurations were compared on the basis of volumetric productivity. The production of laccase and manganese peroxidase by *Panus tigrinus* using both SmF and SSF were tested in a STR, an ALR and a rotary drum reactor (RDR). The highest total enzyme activity was obtained using the RDR, possibly due to the absence of sheer stress, but the volume of processed effluent per unit reactor volume using the liquid submerged cultures (STR and ALR) systems was ten fold higher than in the RDR. However, fair

comparison of the bioremediation capacity could not be made between the different systems since chopped maize stalks were used as the support in the RDR, and while this lignocellulosic material would have simulated the natural habitat of the white rot fungus and possibly have induced ligninolytic enzyme activity, utilisation of the support as a growth substrate could have caused liquid and gas mass transfer restrictions (Rodriguez Couto *et al.*, 2002). The nature of the support is a critical parameter that may influence continuity in the process. One way of overcoming support degradation problems would be to use an inert support.

The application of batch processes for bioremediation applications is somewhat impractical considering the low amounts of enzymes obtained, and therefore, continuous operations are more feasible (Moreira *et al.*, 1998). A continuously operating system for the bioremediation of phenols was demonstrated using a modular capillary membrane reactor (Luke and Burton, 2001). The immobilised fungal biofilm of *N. crassa*, the common pink mould, was tested for its ability to degrade phenol and cresol. The degradative activities were compared to those obtained in non-immobilised cultures, and higher laccase and polyphenol oxidase activities were found in static cultures than in submerged cultures. The value of using a continuous system was confirmed through the increased tolerance of the immobilised *N. crassa* to phenol, where the biofilm was maintained in a viable state for over four months.

In a further example of a WRF culture, *P. chrysosporium* was grown on an inert nylon sponge support in a rotating drum bioreactor, allowing the continuous production of lignin peroxidase at high levels of activity without operational problems (Dominguez *et al.*, 2001). Here, the inertness of the support allowed a focused study of the efficiency of the reactor without interactions of variables related to the composition of the support. The physical features of the nylon sponge permitted good attachment for the fungus, while the continuous rotation provided gentle agitation that prevented clogging of the support. The high lignin peroxidase activity obtained was attributed to the semi-solid state conditions and the high aeration rate, which made more oxygen and nutrients available to the microorganisms.

In studies to maximise enzyme production by WRF in bioreactors, the highest reported laccase production by *T. versicolor* was obtained under solid-state conditions in a tray bioreactor, as compared with an immersion bioreactor and an expanded-bed tubular reactor (Rodriguez Couto *et al.*, 2002). The latter two systems included agitation (mechanical and pneumatic agitation, respectively) that caused shear stress which was inhibitory to the fungus. Optimisation of key parameters such as aeration is important in solid-state fermentation. Here, the tray bioreactor system provided optimal aeration, such that oxygen limitation, at low aeration rates, and fungal overgrowth at high aeration rates were avoided. High laccase activity was also produced in a continuous system by growing *Trametes pubescens* in a STR, although its production was suggested to be repressed by agitation (Galhaup *et al.*, 2003). Laccase production was induced through the addition of copper and its production was increased by continuously feeding low, non-repressing amounts of glucose in a fed-batch cultivation, while maintaining low mechanical agitation at

100 rpm. The effects of shear stress caused by mechanical agitation can be avoided while still maintaining good mixing and mass transfer by using an airlift loop bioreactor, as was demonstrated by Ryan *et al.* (2005). Here, high laccase activity was produced by *T. pubescens* during the bioremediation of a cresylic effluent. For coal biosolubilisation by WRF producing laccase, where the presence of solid particles contribute to shear stress, the ALR may more be feasible than other bioreactor systems in terms of achieving good aeration and mixing, while minimising shear stress. In addition to this, the ALR is simple and has low energy requirements (Christi and Moo-Young, 1987, 1993).

1.5 BIODEGRADATION OF SUBSTRATES SIMILAR TO LOW RANK COAL

This section was included in this literature review because although the mechanisms of coal bioconversion are poorly understood, lignin, humic acids, melanin, polycyclic aromatic hydrocarbons and certain dyes are all compounds that have similar structures to low rank coal, and the bioconversion of these materials can potentially provide supporting theory to explain the mechanism of coal bioconversion.

1.5.1 Lignin bioconversion

The only known microorganisms that have the ability to extensively degrade lignin (to CO₂) are basidiomycetous fungi (Roy and Archibald, 1993; Hofrichter, 2002). Thus, degradation of lignin in wood and in soil has been observed in wood-colonizing fungi causing white rot, and in litter-decomposing fungi, respectively (Hofrichter, 2002). A major application of the degradation of lignin by white rot fungi is the delignification and bleaching of kraft pulp in the paper industry. White rot fungi have been extensively studied in this regard, and among these, *P. chrysosporium* and *T. versicolor* are well characterised (Kondo *et al.*, 1999, Bourbonnais and Paice, 1990; Hammel and Moen, 1991; Roy and Archibald, 1993; Addleman and Archibald, 1993). The key enzymes secreted by white rot fungi (WRF) that are involved in lignin degradation are extracellular oxidases: lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) (Li *et al.*, 2001; Archibald *et al.*, 1997; Reid, 1998).

Mineralisation of lignin requires not only oxidative breakdown of the polymer, but also reductive reactions, causing ring cleavage of aromatic nuclei. The system is complex and a number of proposed mechanistic pathways exist. Certain fungi secrete only one or two of the ligninolytic enzymes, and in fungal strains where more than one enzyme is present, differences in peak production times suggest a synergistic function (Tuor, 1995). The involvement of so-called feed-back type enzymes such as glucose oxidase, aryl alcohol oxidase, veratryl alcohol oxidase, cellobiose dehydrogenase and cellobiose:quinone oxidoreductase have also been suggested (Leonowicz, 1999). Their role is to link metabolic pathways during the biodeterioration of wood. As an example, glucose oxidase provides hydrogen peroxide from the break down of glucose for lignin and manganese peroxidase, and reduces quinones, yielded by laccase,

to form phenols. The phenols become substrates for enzymes such as catechol 1,2-dioxygenase, which catalyse the cleavage of aromatic rings to form keto acids that are taken up in the Krebs cycle (Leonowicz, 1999).

Fungal degradation of lignin results mainly in the formation of aromatic carboxylic acids of low molecular weight. These molecules can be taken up into cells where metabolism to carbon dioxide can occur. *In vitro*, polymerisation reactions may occur due to coupling of phenoxy radicals producing quinones. However, in lignin degradation in wood, cellobiose dehydrogenase and cellobiose:quinone oxidoreductase are present and function as phenoxy radical-reducing enzymes. These redox enzymes may be used *in vivo* to regulate lignin depolymerisation reactions (Ander and Marzullo, 1997).

Since the molecular size of the ligninolytic enzymes does not allow their penetration of wood, the involvement of low molecular weight intermediates such as veratryl alcohol, oxalate and 3-hydroxyanthranilic acid, have been investigated for their ability to act as diffusible oxidants, assisting with degradation of lignin (Leonowicz, 1999), as discussed in greater detail in Section 1.3.3.

1.5.2 Polycyclic aromatic hydrocarbon biodegradation

Polycyclic aromatic hydrocarbons (PAHs) constitute a large, diverse group of organic molecules and are more simply known as polyarenes. These ubiquitous pollutants, found in sediments, soil, air, surface water, and plant and animal tissue, originate from the burning of crude oil, coal, oil shale, and other processes in the oil industry (Brack and Schirmer, 2003; Mi-Sun *et al.*, 1998; Harvery, 1997). Some PAHs are released into the environment during the preparation and transportation of coal. In a study undertaken by Zhao *et al.* (2000), eight bituminous coals were tested for the presence of free PAHs by extracting the coal with dichloromethane. The GC-MS results showed that 20 different PAHs were present, of which naphthalene, acenaphthalene, phenanthrene, anthracene, pyrene and beno[b]pyrene were the main constituents.

Increased interest in the use of microorganisms to decontaminate PAH-polluted sites has arisen from the discovery of the mutagenic and carcinogenic properties of these persistent environmental pollutants (Zhao *et al.*, 2000; Baborova *et al.*, 2006). Early bioremediation projects involved the investigation of indigenous bacterial flora to degrade PAHs (Andersson and Henrysson, 1996). Degradation pathways of simple PAHs are well characterised in bacteria, and genes for the initial step in the degradation of naphthalene, phenanthrene and dibenzothiophene have been cloned and sequenced in many strains (Harayama, 1997). However, high molecular mass PAHs are not readily degradable due to low solubility, and restricted transport through cell membranes. Apart from the low solubility and hydrophobicity of PAHs, their high partition coefficients cause strong sorption to surfaces of particles. The addition of surfactants to increase

bioavailability has been investigated in this regard (Doong and Lei, 2003), but inhibitory effects of surfactants at high concentrations occurred (Harayama, 1997).

Extracellular oxidation by oxidative enzymes presents an attractive alternative means of degradation of PAHs. This has led to the investigation of WRF to degrade PAHs, since they are the most efficient producers of extracellular oxidative enzymes (Eggen and Majcherczyk, 1998), and their lignin-degrading systems enable degradation of a wide range of high molecular weight pollutants (Canet *et al.*, 2001). Degradation of PAHs by WRF has been demonstrated in submerged cultures and also in contaminated soils (Kim *et al.*, 1998; Eggen and Majcherczyk, 1998).

P. chrysosporium has been used as a model organism for degradation of PAHs, but recently several other fungal genera such as *Trametes*, *Pleurotus*, *Bjerkandera*, *Ramaria* and Agaricales have been investigated (Andersson and Henrysson, 1996). LiP from *P. chrysosporium* catalyses one- electron oxidation of PAHs to produce unstable aryl cation radicals. MnP is thought to oxidise PAHs with high ionisation potentials by lipid peroxidation-based co-oxidation reactions. In some fungi that do not possess the ligninolytic enzymes, initial attack on PAHs is achieved by the action of cytochrome P450 monooxygenases, and it is possible that these play an important role in PAH degradation by WRF, since their activity was detected in *P. chrysosporium* when converting hydroxylated benzo[*a*]pyrene (Harayama, 1997).

1.5.3 Biodegradation of humic acid

Humic acids are highly pigmented, organic compounds that are formed through transformation of substances derived from decaying plant and animal tissue through microbial activity. They are extractable from soils and sediments by using bases, and may also be separated from natural brackish water environments (Beckett, 1987; Steffen *et al.*, 2002). Humic substances are divided into 3 groups: 1) humic acids, which are soluble in alkali only 2) fulvic acids, which are soluble in alkali and acid and 3) humin, which is insoluble in both alkali and acid.

Since lignin is the major parent material in the formation of humic substances, it seemed pertinent that humic acid degradation by white rot fungi (WRF) be investigated. The conversion of high molecular weight humic acid to low molecular weight fulvic acids and carbon dioxide by the soil colonizing basidiomycete, *Collybia dryophila*, was demonstrated by Steffen *et al.* (2002), and the depolymerisation and mineralisation of humic acids was shown to be facilitated by manganese peroxidase and laccase.

Humic acids are also extractable from low rank coal, and therefore, its depolymerisation has been used as an indicator of coal depolymerising activity (Fakoussa and Frost, 1999; Willmann and Fakoussa, 1997; Henning *et al.*, 1997; Hofrichter and Fritsche, 1996). *Nematoloma frowardii* (subsequently reclassified as *Phlebia* sp. Nfb19), *P. chrysosporium* and *T. versicolor* are among the many WRF which have displayed

the ability to bleach coal-derived humic acids. Granit *et al.* (2007) were the first to report the isolation of WRF from thermophilic compost that had the ability to degrade lignin and humic acid, signifying their role in humus turnover. The use of humic acid as a model compound for screening microorganisms for the ability to bioconvert low rank coal is, therefore, a useful tool.

1.5.4 Biodegradation of dyes and industrial effluents

As much as 10-20% of dyes are lost into wastewater during dyeing processes, and wastewaters from the textile, food, paper, and cosmetic industries are highly coloured due to the presence of complex synthetic dyes (Soares *et al.*, 2001; Robinson *et al.*, 2001). These include heterocyclic, azo- and triphenyl methane dyes that are often resistant to biological treatment processes (Ollikka *et al.*, 1993), due to their recalcitrant nature and intended capacity to withstand destruction by light, water, and oxidising agents (Robinson *et al.*, 2001).

Biotechnological approaches for the remediation of these toxic compounds include the use of bacteria and fungi. Anaerobic degradation, however, has been reported to produce carcinogenic and/or mutagenic products (Valli *et al.*, 1992; Chander and Arora, 2007). The ability of WRF to degrade dyes has been known for a long time (Glenn and Gold, 1983). The versatility with which these fungi can degrade a broad range of recalcitrant dyes has been demonstrated (Field *et al.*, 1993; Barr and Aust, 1994) and is attributed to their complex lignin- degrading enzyme systems. In fact, various azo dyes have been used in screening procedures for detection of such enzymes and measurement of degradative capabilities of microorganisms (Pasti-Grigsby *et al.*, 1994; Glenn and Gold, 1983; Field *et al.*, 1992; Moreira *et al.*, 2001; Kiinskinen *et al.*, 2004).

The PAH-degrading capability of newly isolated WRF was screened using the common polymeric anthraquinone dye, Poly R-478 where the rate of dye decolourisation was highly correlated to the biodegradation of PAHs (Field *et al.*, 1992). The degradation of ten different dyes, belonging to the azo, triphenyl methane, heterocyclic, and polymeric type groups, by *P. chrysosporium*, was reported by Ollikka *et al.* (1993), and varying specificities of lignin peroxidase isoforms towards the dye substrates was demonstrated. Conversely, *T. versicolor* mutants bearing low manganese peroxidase and laccase activity showed decreased abilities to degrade Poly B-411, Poly R-478, and phenol red (Addleman *et al.*, 1995). Soares *et al.* (2001) investigated the ability of laccase to decolourise another anthraquinone-type dye, Remazol Brilliant Blue (RBBR), and concluded that the inclusion of a redox mediator was necessary for dye decolourisation by commercial laccase.

Screening for laccases from new fungal isolates with potential industrial applications, such as textile dye decolourisation, and delignification of pulp and effluent detoxification, using Poly R-478 and RBBR, was conducted by Kiinskinen *et al.* (2004). Four novel thermostable laccases were identified. Recently, the decolourisation of Indian industrial dyes by some less commonly studied WRF (*Dichotomus squalens*,

Daedalea flavida, *Irpex flavus* and *Polyporus sanguineus*), was successfully demonstrated (Chander and Arora, 2007). Here, cell free enzyme extracts were used to overcome the drawback of dye adsorption to fungal mycelia. In conclusion, the degradation of polymeric dyes, which are often recalcitrant to biodegradation and, like humic acids, require an extracellular mechanism of attack by microorganisms, may be used as an initial screening assay for the production of ligninolytic enzymes, and may provide information on the potential of microorganisms to depolymerise of low rank coal.

1.6 RESEARCH OBJECTIVES

The bioconversion of South African sub-bituminous coal to produce value-added carbon intermediates has not been investigated before, despite the high level of coal mining activity in the country. This bioconversion could be applied to coal that is not used to generate energy and coal fines that are wastes in the coal industry. Thus, the overall aim of this project was to investigate the bioconversion of sub-bituminous coal to produce low molecular weight carbon intermediates that could potentially serve as a substrate for the production of value added compounds.

The first objective of this study was, therefore, to acquire microbial strains that had the potential to degrade low rank coal. Following from this, the second objective was to choose the best strain capable of coal bioconversion and to identify the degradation products.

A better understanding of the degradation pathways was necessary in order to optimise the production of desirable intermediates. Thus, the third objective was to investigate the bioconversion of coal by enzyme preparations of the chosen strain to identify phenolic intermediate products.

Lastly, the fourth objective was to investigate the most appropriate bioreactor configuration for the bioconversion of coal by *Trametes pubescens* (the chosen fungal strain), and to use this system to provide a preliminary kinetic analysis of the yields of the coal bioconversion process. Since coal is an insoluble substrate, solid state cultivations in column reactors were tested and compared with submerged cultures that are most commonly reported in the literature.

1.7 THESIS STRUCTURE

An extensive review of the literature on the state of the art of coal bioconversion is presented in **Chapter 1**. The starting point of the experimental work was to obtain a collection of microbial strains that could be screened for coal bioconversion activity. The choice of microorganisms, all fungi, was largely based on their lignin bioconversion capabilities, as reported in literature. Once the fungal strains were acquired (from international culture collections and environmental isolates), selective solid media was used as a screening tool to identify strains that showed the ability to produce ligninolytic enzymes, depolymerise lignin and coal-derived humic acids, and to utilise coal model compounds as a carbon and energy source. This work is reported in **Chapter 2**, together with elemental and proximate analysis of the sub-bituminous coal (and similar substrates) used in the bioconversion studies carried out in shake flask cultures.

Following this, it was decided that the focus of subsequent experiments would be to investigate coal bioconversion in shake flask cultures with the best strains, identified from the solid media screening assays. The environmental isolates were of particular interest because they were newly isolated in the Western Cape, and there existed the possibility of discovering novel coal bioconversion activities in these strains. Thus, *Trichoderma atroviride* ES11, a new isolate of this species, having shown the ability to grow on coal as the sole carbon source, was among the strains chosen for liquid culture experiments. The work with strain ES11 was performed in collaboration with co-workers at the University of the Western Cape, and is the subject of **Chapter 3**.

At this stage, the focus of the coal bioconversion experiments changed to investigations involving *T. versicolor* and *T. pubescens*, because the desired lower molecular mass carbon units were not obtained with ES11. Of the fungal strains obtained from culture collections, the best results from the solid media screening assays were obtained with *T. versicolor* and *T. pubescens*. These strains are good producers of laccase and their ability to depolymerise coal-derived humic acids in the absence of an additional, easily utilisable carbon source prompted the investigation of coal bioconversion using these strains in shake flask cultures. Analyses of the coal bioconversion products were also performed. Coal bioconversion by *T. versicolor* and *T. pubescens* in shake flasks is presented in **Chapter 4**.

All the investigations conducted to this point were mainly exploratory, and preceded the reactor work which is discussed in **Chapters 5** and **6**, with the most important data reported in Chapter 6. Several reports on coal bioconversion with *T. versicolor* are found in literature, and hence, the novelty of the research presented in this thesis lay in coal bioconversion by *T. pubescens*. A detailed understanding of the coal bioconversion pathway of *T. pubescens* was investigated using a cell-free filtrate. This is described in Chapter 5. In Chapter 6, a comparison of four different reactor configurations, used in the

bioconversion of coal by *T. pubescens*, is presented. In addition to this, and more importantly, the most suitable bioreactor system was used to provide a preliminary kinetic analysis of coal bioconversion process yields.

Finally, in **Chapter 7**, overall conclusions are drawn, and recommendations are made regarding the improvement of coal bioconversion technologies, and focal points of future research are proposed.

University of Cape Town

CHAPTER 2: ANALYSIS OF COAL AND SCREENING OF FUNGAL STRAINS FOR COAL BIOCONVERSION ACTIVITY

2.1 INTRODUCTION

The work described in this chapter includes 1) the characterisation of the coal and structurally related compounds, based on elemental and proximate analyses, and 2) screening of fungal strains for their ability to bioconvert low rank coal using solid media assays, based on production of ligninolytic enzymes, depolymerisation of lignin and humic acids, and utilisation of coal model compounds as a carbon and energy source.

There are great variations in the types of coal found in various areas of the world, and hence major differences in the types of coals used in coal bioconversion research (Section 1.2.2, Table 1.1). It was therefore helpful to provide information on the composition of the coal to be studied here, in order to effectively compare coal bioconversion data found in literature. Further, the reactivity of microorganisms towards coal and the resulting products is largely dependent on the characteristics of the starting coal material (Faison, 1991). The key feature of low rank coals is the presence of ether linkages and carboxyl groups, which make the coal more reactive and, therefore, more amenable to biological attack (Faison, 1991), and the soft coals (mostly lignite) have been studied in most bioconversion processes for this reason (Section 1.3 Table 1.3). However, the present study included harder, sub-bituminous coal because its reserves are found in much larger quantity in South Africa than lignite.

The bioconversion of coal occurs by a few different mechanisms, namely, biosolubilisation, biodepolymerisation and bio-utilisation (Klein *et al.*, 1999; Hofrichter and Fakoussa, 2001). The catabolism to lower molecular mass units (depolymerisation) can only be achieved by non-specific, indirect and extracellular enzymatic attack on the coal (Fakoussa and Hofrichter, 1999; Catcheside and Ralph, 1999; Klein *et al.*, 1999; Hofrichter and Fakoussa, 2001).

As a means of identifying microbial strains capable of coal bioconversion, the extracellular production of ligninolytic enzymes (LiP, MnP and laccase) allows for simple solid media screening assays using indicator compounds which are structurally similar to lignin, and represent coal substructure model compounds. Screening processes employing the use of polymeric dyes are common due to the simplicity of the assay, and polymeric dyes, such as Poly R-478, are considered to be good indicators of ligninolytic activity because dye decolourisation *in vivo* coincides with the onset of lignin metabolism in white rot fungi (Glenn and Gold, 1983; Gold *et al.*, 1988). Poly R-478 (violet anthraquinone) is a stable polymeric dye that has an average molecular mass of 40 kDa, and the production of ligninolytic enzymes is evidenced by the formation of decolourised halos, which occur along the periphery of mycelial growth (Gold *et al.*, 1988).

This approach was used in this study in order to identify microbial strains that produce extracellular ligninolytic enzymes, and have the ability to potentially depolymerise coal macromolecules.

Poly-8-hydroxyquinoline is a polymer of 8-hydroxyquinoline, recently produced by the oxidative polymerisation by laccase (Ncanana and Burton, 2007). Its average molecular weight is 789. In this study, this compound was chosen as an indicator since it can be depolymerised back to the monomeric unit, but further polymerisation may also be observed, when laccase is present, by the production of an orange colouration. The growth of fungi on model compounds such poly-8-hydroxyquinoline and phenanthrenequinone (Figure 2.1) suggests that these organisms must be able to enzymatically cleave bridging groups which contribute to the structural integrity of coal, or be able to utilise the aromatic compounds likely to be present in the mobile phase of coal, respectively (Ralph and Catcheside, 1994). Furthermore, compounds such as phenanthrenequinone and poly-8-hydroxyquinoline or structurally related compounds are likely to be present in the coal mobile phase. This could provide an explanation for the ability of some isolates to grow on coal-containing agar, without being able to depolymerise the coal matrix (Ralph and Catcheside, 1994). This is a point to note when using enrichment cultures in order to isolate coal-degrading microorganisms. Nonetheless, investigation of a microorganism's ability to utilise such aromatic compounds can help in the elucidation of coal conversion mechanisms.

The use of solid media screening by means of enrichment culture or addition of solid low rank coal to surface cultures has also been reported (Cohen and Gabriele, 1982; Scott *et al.*, 1986; Catcheside and Mallet, 1991; Crawford and Gupta, 1991; Ralph and Catcheside, 1993, 1994; Hölker *et al.*, 1995; Hofrichter *et al.*, 1997; Labardo *et al.*, 1999). Biosolubilisation of low rank coal to form liquid products on surface cultures does not necessarily imply depolymerisation and/or the action of ligninolytic enzymes; other factors such as the action of alkaline substances, chelators and even hydrolytic enzymes are possible (as discussed in Section 1.3.1). For this reason the use of lignin and coal-derived humic acid bleaching in solid media provides an optimal method for the detection of desired depolymerisation activity. Breakdown of the coal structure is evidenced by a bleaching effect around the mycelial growth area of certain fungi, taken to indicate the activity of extracellular enzymes (Hofrichter and Fritsche, 1996).

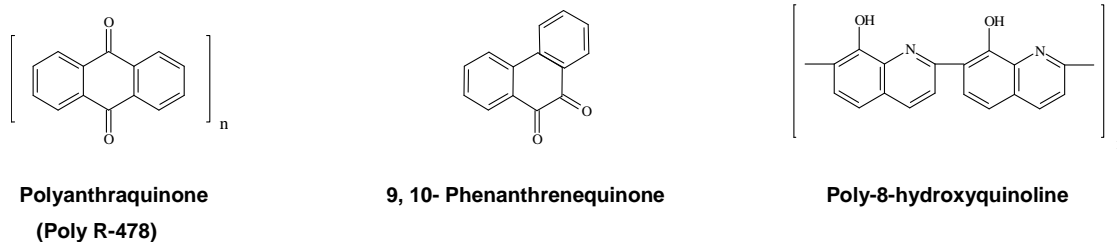


Figure 2.1 Chemical structures of polymer subunits of Poly R-478 and Poly-8-hydroxyquinoline, and 9,10-phenanthrenequinone

2.2 MATERIALS AND METHODS

2.2.1 Source and composition of coal and related compounds

Sub-bituminous coal was kindly provided by Mr Johan van Dyk, SASOL (2001). Lignite (brown coal) was sampled in New Zealand, and was a gift from Prof Donald Cowan. Lignin (Indulin AT), from Kraft pine, was obtained from MeadWestvaco Corporation (USA) as a gift. Asphaltene, derived from the heptane and pentane insoluble fraction of Canadian A heavy oil, was donated by Prof Francis Arnold, Caltech (USA). Humic acid was purchased from FLUKA, (a subsidiary of Sigma-Aldrich, Switzerland).

Microanalysis of these substrates was conducted in the Department of Chemistry, University of Cape Town, using a Thermoflash 1112 series C, H, N, S and O analyser with combustion analysis in pure oxygen and GC column separation using helium gas. Compounds were detected using differential thermal conductivity. Proximate analysis of the sub-bituminous coal was performed by WITLAB (Pty) Ltd (Coal and Mineral Analysts, Witbank, South Africa).

2.2.2 Acquisition and screening of microorganisms for the biodegradation of low rank coal

Selected fungal strains were obtained from international culture collections, including *Neurospora crassa* #2054, *Trametes versicolor* #3845 (PPRI, Pretoria, RSA), *Trametes pubescens* # 696.94 (CBS, Netherlands), *Nematoloma frowardii* #11239, *Pleurotus ostreatus* #1020, *Pycnoporus cinnabarinus* #1184, *Phlebia radiata* # 5111 and *Bjerkandera adusta* # 4710 (DSMZ, Germany). *Nematoloma frowardii* was reclassified as *Phlebia* sp. *Nfb19* in 2008 (Hildén *et al.*, 2008).

Six new strains (ES04; ES06; ES11; ES13A; ES13B; ES17) were also obtained through collaboration with the Advanced Research Centre for Applied Microbiology (ARCAM), University of the Western

Cape. These strains were isolated from soil, wood and coal at various environmental sites in the Western Cape, South Africa, by direct isolation on malt extract agar supplemented with 1% particulate low rank coal. All strains were routinely sub-cultured and maintained on 3% malt extract agar and stored at 4°C.

The substrates: 0.008% (wt/vol) Poly R-478 (Sigma); 0.1% Poly-8-hydroxyquinoline (gift from S. Ncanana (Ncanana and Burton, 2007)); 0.1% phenanthrenequinone (Sigma); 0.75% - 3.0 % Indulin AT (lignin) (Meadwestvaco); 0.1% lignite (sampled in New Zealand) and 0.1% humic acid (Fluka) were added to solid media as indicators to detect fungal strains capable of producing ligninolytic enzymes and/or the ability to utilize these compounds for growth, when present as the sole carbon source. The media compositions are shown in Table 2.3. Poly R-478, dissolved in water; poly-8-hydroxyquinoline, dissolved in 1, 4-dioxane and phenanthrenequinone, dissolved in acetone, were added after filter sterilization. Malt extract agar, yeast extract, agar bacteriological and glucose were obtained from Merck, SA. Benomyl (Fluka), a benzimidazole fungicide that allows selection of wood decay fungi (D'Annibale *et al.*, 2006), and suppresses mycelial growth by preventing nuclear division, was added to the media prior to autoclaving, to facilitate visualisation of decolourisation halos (Chiocchio *et al.*, 2000). The plates were inoculated aseptically by placing 1cm²-mycelial plugs in the centre of each plate. The plates were incubated at 28 °C. Decolourisation was determined by measuring the diameter of the halo.

2.3 RESULTS AND DISCUSSION

2.3.1 Analysis of coal

In order to characterise substrates to be used in the bioconversion studies, microanalysis was conducted on lignite and sub-bituminous coal, and structurally related compounds, including lignin, humic acid and asphaltene. The results illustrated an expected increasing carbon content, and a concomitant decreasing oxygen content, from lignite to asphaltene, as the degree of the coal condensation increased (Table 2.1).

Table 2.1 Elemental composition of coal and related compounds

	Lignin	Humic acid	Lignite	Sub-bituminous coal	Asphaltene
%C	60.0	42.86	50.46	59.13	78.18
%H	5.86	4.32	4.71	3.66	7.69
%N	0.76	0.68	0.55	1.24	0.91
%S	1.67	0.60	1.59	0.61	7.54
%O	28.43	31.54*	27.93	14.84	1.68

* By difference (ash content = 20%)

The South African sub-bituminous coal used in this study would be classified as lignite, based on the above elemental composition, and according to conventional classification of coal (reviewed by Klein *et al.*, 1988; Faison, 1993; Hodek, 1994). The technological behaviour of coal, however, cannot be determined from the elemental composition alone (Snyman, 1998). A proximate analysis of coal, expressed as moisture, ash, volatile matter and fixed carbon content (see Section 1.2.2, Figure 1.2), provides a more precise means of coal classification, which is of particular importance in the commercial production of coal. The result from the proximate analysis of the South African sub-bituminous coal is shown in Table 2.2. The fixed carbon content was found to be 49.7%, which was lower than the results of the carbon content determined by elemental analysis, indicating that some carbon was present as volatile matter (i.e. part of the mobile phase of coal). The ash content, which provides an indication of the mineral content of the coal, was found to be 20.9%, indicative of a lower grade coal. Low rank coals can also be distinguished from hard coal by monitoring the extractability of humic acids by treatment with an alkaline solution such as potassium hydroxide (Klein *et al.*, 1988). Humic acids are not extractable from sub-bituminous coals because they are polymerised; the South African sub-bituminous coal was not soluble in sodium hydroxide solution, confirming its classification.

For the purpose of this study, characteristics such as inherent moisture content provided information regarding the susceptibility of the coal to biological attack. The inherent moisture content is considered to be a reliable indicator of the internal porosity of a coal (Snyman, 1998), and therefore can be taken as an indication of the relative susceptibility to microbial entry. Thus, filamentous fungi and actinomycetes may colonise coal by mechanical penetration of these pores (Faison, 1993). The inherent moisture content of the South African sub-bituminous coal was 4.3%, which is low for a sub-bituminous coal in terms of the conventional classification (Faison, 1993), and suggested that the South African sub-bituminous coal may be less susceptible to biological attack than expected.

Table 2.2 Proximate analysis of South African sub-bituminous coal

Description	
Inherent moisture content	4.3 %
Ash content	20.9 %
Volatile matter	25.1 %
Fixed carbon content	49.7 %
Calorific value	22.66 MJ/Kg

2.3.2 Screening of fungal strains on solid media

Screening of microorganisms capable of the bioconversion of sub-bituminous coal was carried out using solid media assays. The ability to bioconvert coal was based on three assays: i) The decolourisation of the Poly R-478, which would indicate the production of extracellularly-produced ligninolytic enzymes, 2) the decolourisation (or bleaching) of lignin and/or coal-derived humic acids, which would indicate the ability of the microorganism to potentially depolymerise coal macromolecules, and 3) growth on model compounds, poly-8-hydroxyquinoline and/or phenanthrenequinone, which would show the ability of the microorganism to utilise the model compound and thus coal, as a carbon and energy source. The fungal strains that were screened were obtained from the Dutch (CBS) and German (DSMZ) culture collections, and some wild-type isolates from environmental samples (Silva-Stenico, 2007).

The wild-type fungal strains were isolated from soil, wood and coal in the Western Cape (South Africa) by direct isolation on a minimal salts solid medium containing 1% (w/v) coal as the sole carbon source (Silva-Stenico, 2007). Subsequent analysis of genomic DNA from the environmental isolates revealed the identity of the strains as: ES04 – *Bipolaris spicifera*; ES06 – *Alternaria tritiana*; ES11 – *Trichoderma atroviride*; ES 13(A+B) – *Alternaria alternata* and ES17 – *Penicillium chrysogenum*. This work was performed in collaboration with Dr M.E. Silva-Stenico at ARCAM, University of the Western Cape. A summary of the screening assay results is presented in Table 2.3.

2.3.2.1 Detection of ligninolytic enzymes using Poly R-478

In screening for ligninolytic enzyme activity in fungal strains based on growth and dye decolourisation, positive reactions, as indicated by the decolourisation of Poly R-478, were achieved with *T. versicolor*, *T. pubescens*, *N. frowardii* and *P. cinnabarinus*. *P. ostreatus* and *B. adusta* were not able to grow on the plates but decolourisation of the dye was visible around the inoculation plug, suggesting growth inhibition although extracellular ligninolytic enzymes were present. None of the six environmental isolates decolourised Poly R-478, although they all showed the ability to grow on the plates, with the exception of ES 17 (Table 2.3). The environmental isolates belong either to the Ascomycete or Deuteromycete group of fungi, and therefore their ability to grow on solid media containing benomyl was unusual. *Neurospora crassa*, also belonging to the Ascomycete group, and known to produce laccase and polyphenol oxidase, (Luke and Burton, 2001) was unable to grow or decolourise Poly R-478. *N. crassa* produces polyphenol oxidase solely as an intracellular product, but produces laccase predominately as an extracellular product (Luke and Burton, 2001).

In a screening programme used to detect novel laccase-producing microbes, Kiinsken *et al.* (2004) isolated 26 fungal strains which showed positive reactions on indicator plates containing Remazol Brilliant Blue R, Poly R-478, guaiacol or tannic acid. Isolates belonging to the *Trichoderma* species (*T. atroviride* and *T. harzianum*) which do not commonly produce ligninolytic enzymes, produced positive reactions on

indicator plates (Kiinsken *et al.*, 2004). *T. atroviride* also produced extracellular laccase when glucose was present as an additional carbon source during the bioconversion of lignite (Hölker *et al.*, 1999). However, in this study, the environmental isolate, *T. atroviride* ES11 was unable to decolourise Poly R-478, suggesting that it did not produce any extracellular ligninolytic enzymes.

The composition of the solid media was then changed, based on experiments reported by Hölker *et al.* (1999), by adding glucose instead of malt extract and by eliminating benomyl. Again, *T. atroviride* ES11 did not decolourise Poly R-478 (Figure 2.2). Thus, contrary to the findings of Kiinsken *et al.* (2004) and Hölker *et al.* (1999), the type of additional carbon source, whether malt extract or glucose, did not have an effect on the decolourisation of Poly R-478 by *T. atroviride* ES11, and the production of extracellular ligninolytic enzymes under the specific assay conditions was not apparent. Specific enzyme activities of *T. atroviride* ES11, grown in liquid culture, were subsequently performed and are discussed in Chapter 3.



Figure 2.2 Growth of *T. atroviride* (ES11) on Poly R-478 plates containing malt extract (row 1) and glucose (row 2) as the carbon source. No decolourisation was apparent regardless of the carbon source added.

Positive decolourisation reactions were obtained with *T. versicolor*, *T. pubescens*, and *N. frowardii* on Poly R-478 plates containing malt extract, and glucose (Figure 2.3). Faster decolourisation was observed in the plates containing glucose. This is probably due to faster nutrient depletion since glucose is more easily metabolised and the production of ligninolytic enzymes, in some instances, occurs under nutrient limiting conditions (Moreira, 2004; Robinson *et al.*, 2001; D'Annibale *et al.*, 2006).

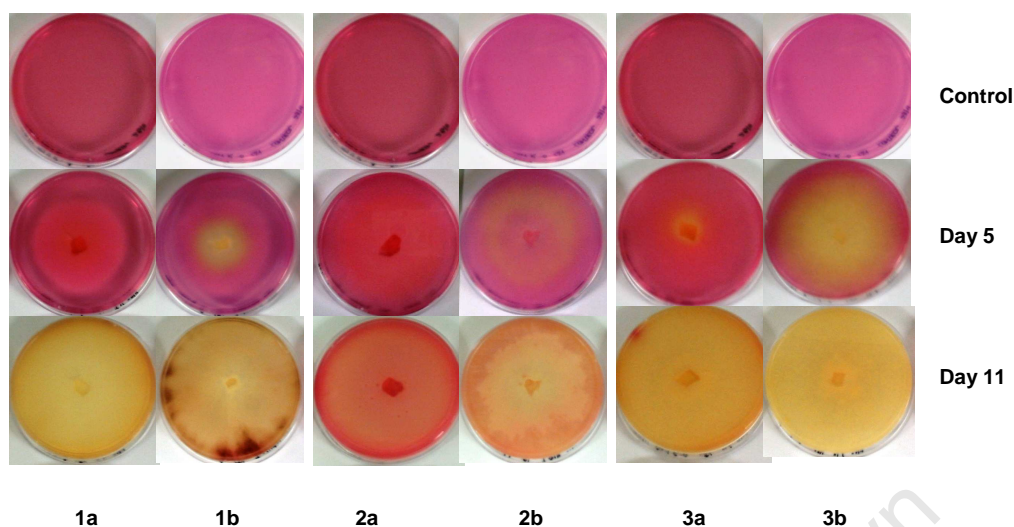


Figure 2.3 Poly R-478 decolourisation by *T. versicolor* (row 1a & b), *T. pubescens* (row 2a and b) and *N. frowardii* (row 3a & b). Plates contained malt extract (row 1a, 2a, 3a) or glucose (row 1b, 2b, 3b) as carbon source.

T. versicolor is one of the most extensively studied white rot fungi, and dye decolourisation by *T. versicolor* has been reported in numerous accounts (Moreira *et al.*, 2004; Soares *et al.*, 2001; Robinson *et al.*, 2001; Swamy & Ramsay, 1999; Addleman *et al.*, 1995 and Field *et al.*, 1992). In this particular strain of *T. versicolor*, laccase is the predominant ligninolytic enzyme (Ryan, 2003) but small amounts of manganese peroxidase have also been detected (data shown later). In the present study Poly R-478 degradation by *T. versicolor* occurred more rapidly than *T. pubescens*, but more slowly than by *N. frowardii*. The faster rate of decolourisation could mean that higher extracellular ligninolytic enzyme activities were produced, and/or that the onset of enzyme production occurred earlier than in the other strains. Thus, analysis of the ligninolytic enzyme activities in liquid culture would be helpful in obtaining more specific information. This was done later and is discussed in Chapter 4 (Section 4.3.2).

The decolourisation of Poly-R478 by these fungal strains, indicating the production of extracellular ligninolytic enzymes, led to the prediction of their potential to depolymerise lignin and coal macromolecules (discussed in the next section), and ultimately to bioconvert native low rank coal. Of these three white rot fungi, *T. pubescens* is the most recently isolated and least characterised strain. However, it has been reported to be an outstanding producer of laccase (Galhaup and Haltrich, 2001). Recently, Osma *et al.* (2007) reported similar results to the current study on the ability of *T. pubescens* to degrade Poly R-478, and a crude laccase extract from *T. pubescens*, grown on banana skins, decolourised another anthraquinonic dye, Remazol Brilliant Blue R (RBBR) (Osma *et al.*, 2006). Based on

these findings, the potential of *T. pubescens* to bioconvert low rank presented a novel research avenue, which later became the focal microorganisms of this research project (Chapters 4, 5 and 6).

Table 2.3 Results of screening of isolates and culture strains using various solid media

#	Indicator/Substrate	Composition	Strains tested	Growth	Decolourisation	Days
1	Poly R-478 (& complex C-source)	0.1% yeast extract	<i>T. versicolor</i>	+++	+++	7
		75 ppm benomyl	<i>N. crassa</i>	-		n/a
		2% malt extract agar	<i>T. pubescens</i>	+++	+++	7
		0.008% Poly R-478	<i>N. frowardii</i>	++	++	10
			<i>P. ostreatus</i>	-	around inoc. plug	n/a
			<i>P. cinnibarinus</i>	++	+	10
			<i>P. radiata</i>	++	++	10
			<i>B. adusta</i>	-	around inoc. plug	n/a
			ES04	++	-	>10
			ES06	++	-	>10
			ES11	++	-	>10
			ES13 A/B	++	-	>10
			ES17	-	-	n/a
2	Poly R-478 (& simple C-source)	0.1% yeast extract	<i>T. versicolor</i>	+++	+++	11
		2% glucose	<i>T. pubescens</i>	+++	++	10
		1.2% agar agar	<i>N. frowardii</i>	+++	+++	10
		0.008% Poly R-478	ES11	+++	-	5
3	Lignin (& complex c-source)	2.25% Malt extract	ES04	+++	-	11
		0.75% Lignin	ES06	+	-	11
			ES11	+++	-	11
			ES13B	++	-	11
			ES17	-	-	11
			<i>T. versicolor</i>	+++	-	11
		1.5% malt extract	ES04	+++	-	11
		1.5% lignin	ES06	+	-	11
			ES11	++	-	11
			ES13B	+++	-	11
			ES17	-	-	11
			<i>T. versicolor</i>	+++	-	11
		0.75% malt extract	ES04	+++	-	11
		2.25% lignin	ES06	+	-	11
			ES11	++	-	11
			ES13B	+++	-	11
			ES17	-	-	11
			<i>T. versicolor</i>	+++	-	11

Table 2.3 (Continued)

#	Indicator/Substrate	Composition	Strains tested	Growth	Decolourisation	Days
4	Lignin (sole C-source)	0.75% lignin	ES04	+++	-	11
		2.4% agar agar	ES06	+	-	11
			ES11	++	-	11
			ES13B	+++	-	11
			ES17	+	-	11
			<i>T. versicolor</i>	-	++	11
		1.5% lignin	ES04	+++	-	11
		2.4% agar agar	ES06	+	-	11
			ES11	++	-	11
			ES13B	+++	-	11
			ES17	+	-	11
			<i>T. versicolor</i>	-	+	11
		3.0% lignin	ES04	+++	-	11
		2.4% agar agar	ES06	+	-	11
			ES11	++	-	11
			ES13B	+++	-	11
			ES17	+	-	11
			<i>T. versicolor</i>	-	+	11
5	Lignin (sole C-source)	0.1% yeast extract	ES11	+++	-	11
		0.1% lignin*	<i>T. pubescens</i>	+++	+++	18
		1.2% agar agar	<i>T. versicolor</i>	+	+	11
			<i>N. frowardii</i>	+++	-	11
6	Lignin (sole C-source) (pH adjusted)	0.1% yeast extract	ES11	++	-	10
		0.1% lignin*	<i>T. pubescens</i>	+	pol/depol (pic) [§]	10
		1.2% agar agar	<i>T. versicolor</i>	+	pol/depol (pic) [§]	10
			<i>N. frowardii</i>	-	-	10
7	Lignite (sole C-source)	0.1% yeast extract	ES11	+++	-	11
		0.1% lignite*	<i>T. pubescens</i>	+++	++	11
		1.2% agar agar	<i>T. versicolor</i>	+	+	5
			<i>N. frowardii</i>	+++	+++	11
8	Lignite (sole C-source) (pH adjusted)	0.1% yeast extract	ES11	+++	-	10
		0.1% lignite*	<i>T. pubescens</i>	+	pol/depol (pic) [§]	10
		1.2% agar agar	<i>T. versicolor</i>	+	pol/depol (pic) [§]	10
			<i>N. frowardii</i>	-	-	10
9	Poly-8-hydroxy-quinoline (sole C-source)	0.1% yeast extract	ES11	++	-	11
		0.1% P-8-HQ [#]	<i>T. pubescens</i>	+++	++ (orange)	11
		1.2% agar agar	<i>T. versicolor</i>	++	++ (orange)	11
			<i>N. frowardii</i>	+++	-	11
10	Phenanthrene-quinone (sole C-source)	0.1% yeast extract	ES11	++	-	11
		0.1% anthraquinone ^{e^}	<i>T. pubescens</i>	+++	++	11
		1.2% agar agar	<i>T. versicolor</i>	++	-	11
			<i>N. frowardii</i>	+	-	11

Table 2.3 (Continued)

#	Indicator/Substrate	Composition	Strains tested	Growth	Decolourisation	Days
11	Humic acid (sole C-source) (pH adjusted)	0.1% yeast extract	ES11	+++	-	10
		0.1% humic acid*	<i>T. pubescens</i>	+	pol/depol (pic) [§]	10
		1.2% agar agar	<i>T. versicolor</i>	+	pol/depol (pic) [§]	10
			<i>N. frowardii</i>	-	-	10
12	Positive control	0.1% yeast extract	ES11	++	n/a	4
		1.2% agar agar	<i>T. pubescens</i>	+	n/a	4
			<i>T. versicolor</i>	+	n/a	4
			<i>N. frowardii</i>	+	n/a	4

* dissolved in NaOH # dissolved in dioxane ^ dissolved in acetone § polymerisation/depolymerisation - see Figure

+ (0-15 mm) ++ (16-25 mm) +++ (26-40 mm) - radial growth

2.3.2.2 Depolymerisation activity detected by bleaching of lignin, lignite and humic acid

Fungal strains were next screened for the ability to potentially depolymerise coal macromolecules by using solid media containing lignin, lignite and humic acids. Lignin and coal humic acids cannot be taken up into fungal hyphae due to their size. It was therefore reasonable to assume that growth bleaching observed on plates containing lignin, lignite and humic acid, indicated the breakdown of these macromolecules, and that it occurred as a result of an extracellular enzymatic attack (Hofrichter and Fritche, 1996; Willmann and Fakoussa, 1997; Ralph and Catcheside, 1999).

Degradation of lignin in solid media was tested using four different sets of plates. Firstly, the environmental isolates: *B. spicifera* ES04; *A. tritiana* ES06; *T. atroviride* ES11; *A. alternata* ES13B and *P. chrysogenum* ES17, and *T. versicolor* (as a benchmark) were grown on solid media containing varying concentrations of malt extract and lignin. Secondly, the isolates and *T. versicolor* were grown on solid media with lignin as the sole carbon source, at increasing concentrations. Thirdly, a solid medium containing lignin as the sole carbon (0.1%) source and a nitrogen source (yeast extract) was used to test the lignin-degrading capability of strain ES11, *T. versicolor*, *T. pubescens* and *N. frowardii*. Here, the pH of the medium used was very high (~ pH 11) because the lignin was dissolved in sodium hydroxide solution. The fourth set of plates was the same as the third, except that the pH was adjusted to 7.6 with concentrated HCl. (At a lower pH, the lignin precipitated out of solution). In the same way, media containing lignite and humic acid (both highly alkaline and pH adjusted) were used.

All the environmental isolates (*B. spicifera* ES04; *A. tritiana* ES06; *T. atroviride* ES11; *A. alternata* ES13B and *P. chrysogenum* ES17) grew well on lignin agar plates containing malt extract, and on plates where lignin was added as the sole carbon source. Increasing the lignin concentration and decreasing the malt extract concentration seemed to have had no effect on the growth after 11 days of incubation, and the

presence of lignin (as sole carbon source) seemed to have little or no effect on growth of these fungi, but no bleaching was observed. This result confirmed the inability of these microorganisms to decolourise lignin, and was assumed to be due to their inability to produce extracellular oxidative enzymes as was suggested earlier by the negative results obtained with Poly R-478.

T. atroviride ES11 grew well on plates containing lignin, humic acids and lignite that were pH-adjusted, and formed spores rather than a mycelial mat, but did not decolourise these substrates. It should be noted that growth by ES11 on control plates (agar and yeast extract alone) was substantial, suggesting that a large portion of growth on plates containing these substrates was attributable to this. Thus, the ability of strain ES11 to grow on plates containing lignin, lignite and humic acid is not a clear indication of its ability to utilise these compounds as a carbon and energy source for growth.

T. versicolor grew well on plates containing lignin and malt extract agar but did not cause any bleaching. However, bleaching may have been achieved if an extended incubation period was allowed and total nutrient depletion had occurred, possibly resulting in the use of lignin as a carbon source by the fungus. When lignin was added as the sole carbon source, little growth of *T. versicolor* was observed, but evidence of extracellular enzyme activity and the ability to decolourise lignin was confirmed by the formation of halos. This occurred to a greater extent at 0.75% lignin concentration as compared with 1.5 and 3% lignin concentrations (Table 2.3), probably due to inhibition at higher concentrations. Better growth was observed on alkali-solubilised lignin, when yeast extract was added as a nitrogen source. *T. versicolor* produced fine mycelium that was poorly visible but bleaching of lignin did occur. Slightly better growth and decolourisation was obtained on alkali-solubilised lignite plates. The presence of a nitrogen source was therefore necessary for growth and decolourisation of lignin and alkali-solubilised lignite by *T. versicolor*.

T. pubescens and *T. versicolor* grew well on pH-adjusted, humic acid and lignin solid media, but initial formation and subsequent decolourisation of dark brown halos in the agar, was observed. Similar results were obtained with pH-adjusted, lignite media but to a lesser extent (Figure 2.4). The formation and decolourisation of dark brown halos by *T. pubescens* and *T. versicolor* on pH-adjusted lignin and humic acid agar occurred over a period of 10 days. This was possibly due to the release of specific ligninolytic enzymes e.g. laccase and peroxidase, at different times, each catalysing different reactions (Hofrichter and Fritsche, 1997). Since minimal growth was observed on pH-adjusted humic acid, lignin and lignite solid media, the decolourisation observed did not necessarily mean that these macromolecules were broken down, but that oxidation had possibly occurred. In order to confirm that depolymerisation had occurred, analysis of the molecular weight distribution of lignin, lignite and humic acid macromolecules by size-exclusion chromatography, showing a decrease in the average size, would be required (Fakoussa and Frost, 1996). This was not performed here because growth of *T. versicolor* and *T. pubescens* on

alkali-solubilised lignin and lignite (not pH-adjusted), in addition to the decolourisation of these macromolecules, confirmed that depolymerisation had occurred in order to support their growth.

Of all the strains tested, the best growth and bleaching of alkali-solubilised lignin and lignite was achieved by *T. pubescens* (Figure 2.5), confirming that it had the ability to depolymerise these macromolecules. A noteworthy observation was that *T. pubescens* was able to attack large macromolecules in the absence of an easily utilisable carbon source (e.g. glucose), which is unusual (Willmann and Fakoussa, 1997). Generally, an external nutritional source is required by fungi due to their inability to use xenobiotics as a primary carbon and nitrogen source (D' Annibale, 2006; Pointing, 2001), and in the case of lignin, biodegradation was reported to be a co-metabolic event (Kirk and Farrell, 1987). In these experiments, 0.1% (w/v) yeast extract was added to the plates as a source of nitrogen. It is important to note that yeast extract also contains carbon (~20%), which may have been sufficient to support fungal growth. However, better growth of *T. pubescens* was observed on plates containing alkali-solubilised lignin and lignite than in the positive control (without alkali-solubilised lignin or lignite), suggesting that *T. pubescens* was able to use lignin and/or the depolymerised products for growth.

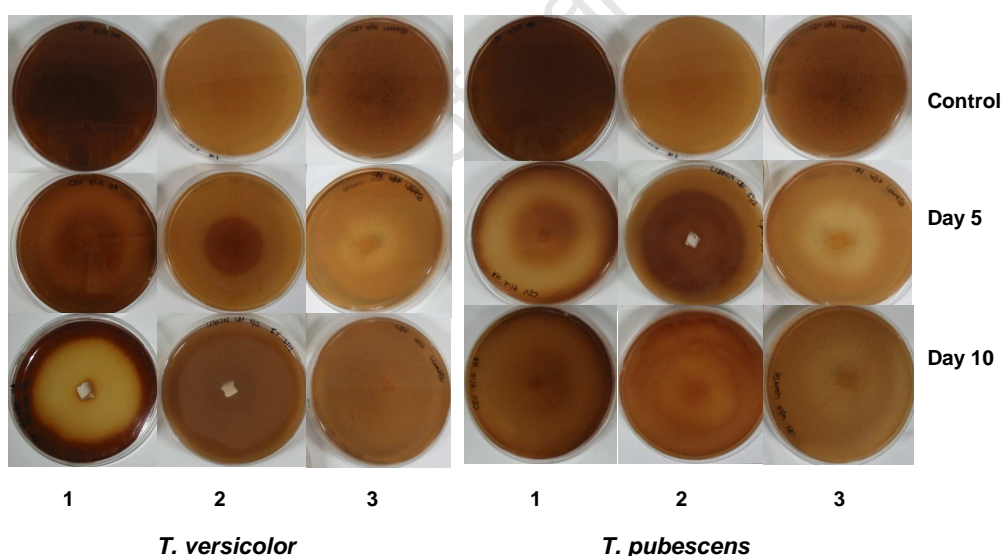


Figure 2.4 Decolourisation of pH adjusted solid medium containing humic acid (row 1), lignin (row 2) and lignite (row 3) by *T. versicolor* and *T. pubescens*

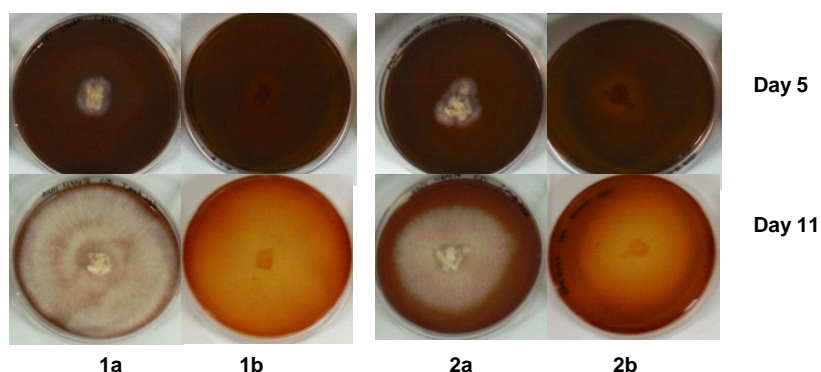


Figure 2.5 Growth and decolourisation of lignin (row 1) and lignite (row 2) by *T. pubescens* ((a) view of plate facing upward, (b) view of plate turned over)

N. frowardii was able to grow on lignin and lignite plates but only beached lignite, and did not grow on or decolourise pH-adjusted humic acid, lignin or lignite agar plates. These results were contrary to the findings of Hofrichter and Fritsche (1996), who developed a specific screening system, using solid media, to select fungi with high potential to depolymerise low rank coals. The bleaching of humic acids from coal was limited to Basidiomycetes, and the most active of their isolates was *N. frowardii*, showing the ability to bleach humic acid and German lignite-derived humic acid. However, the solid medium used in their study also contained glucose and 2, 2-dimethylsuccinate which is an inducer of manganese peroxidase and laccase. Glucose or 2, 2-dimethylsuccinate were not added to the solid medium used in the present study for two reasons: firstly, positive results were obtained with the *Trametes* species tested without further optimisation of the solid medium composition, and secondly, *N. frowardii* has already been extensively studied in the bioconversion of humic acids and coal by Hofrichter and collaborators. Further work on coal bioconversion with *N. frowardii* was therefore not pursued.

2.3.2.3 Utilisation of model compounds, poly-8-hydroxyquinoline and phenanthrenequinone

Poly-8-hydroxyquinoline and phenanthrenequinone were chosen as coal model compounds (discussed in Section 2.1), and these were provided as sole carbon sources in a solid medium containing yeast extract, as nitrogen source, and agar. Poly-8-hydroxyquinoline and phenanthrenequinone were dissolved in 1, 4-dioxane and acetone, respectively, prior to addition to the medium, to give final concentrations of 0.1% of the model compounds in solution.

T. versicolor, *T. pubescens* and *N. frowardii* grew on plates containing poly-8-hydroxyquinoline and phenanthrenequinone, which indicated the ability to utilise these model compounds as a carbon and energy source for growth. However, strain *ES11* showed similar growth on the control and test plates, indicating that its growth may have been supported by the yeast extract and agar. The results obtained in

this study are in agreement with other reports that *T. versicolor* and other *Trametes* species are able to metabolise anthraquinone, which is structurally very similar to phenanthrenequinone. The ability of *N. frowardii* to degrade PAH's has also been demonstrated by Sack *et al.* (1997).

The best growth on phenanthrenequinone and poly-8-hydroxyquinoline was observed in *T. pubescens* (Figure 2.6); it also showed the ability to polymerise poly-8-hydroxyquinoline, indicated by the formation of orange circles (Figure 2.6 (2)). This was also evident in *T. versicolor* cultures, but to a lesser extent. This result was expected, since poly-8-hydroxyquinoline is a product of the polymerisation of 8-hydroxyquinoline by laccase from *T. pubescens* (Ncanana and Burton, 2007), where it was reported that the oxidation resulted in a mixture of polymeric products consisting of oligomers that were orange in colour. In order for *T. pubescens* to have grown on plates containing poly-8-hydroxyquinoline, it must have initially been able to depolymerise the polymer so that monomeric units could be catabolised, and it is possible that some of these monomers were later re-polymerised. It is also possible that the depolymerisation of poly-8-hydroxyquinoline by laccase may be controlled by the addition of an external mediator, similar to ABTS or 1-HBT, as observed in the laccase-ABTS mediated treatment of lignin (Bourbonnaise *et al.*, 1995; Hernandez Fernaund *et al.*, 2006). However, fungi are known to produce their own mediators as a result of metabolism, and in response to the nature of the available substrates (Leonowicz, 1999). This would be a pertinent consideration for later studies on the bioconversion of coal by *Trametes* species.

The ability of *T. versicolor*, *T. pubescens*, *N. frowardii* and *T. atroviride* ES11 (to an extent) to utilise phenanthrenequinone and poly-8-hydroxyquinoline may also be taken as an indication that the growth of these strains in coal bioconversion studies is supported by compounds such as these present in the coal mobile phase (as indicated by volatile matter content of the coal – Section 2.3.1). Thus, the ability of these strains to grow on coal as a sole carbon source is not necessarily an indication of their ability to break down the coal carbon matrix, and should not be used as an indicator of coal bioconversion activity unless supported by additional evidence, *e.g.* size-exclusion chromatography showing a decrease average molecular mass of coal macromolecules.

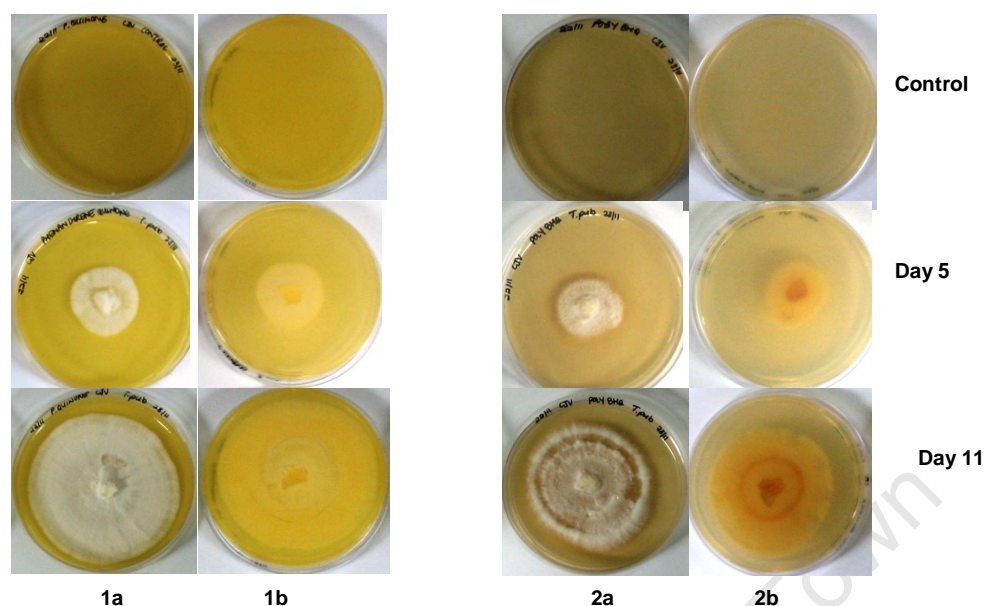


Figure 2.6 Growth of *T. pubescens* on phenanthrenequinone (1) and poly-8-hydroxyquinoline, showing orange colouration which was indicative of a polymerisation reaction (2) ((a) view of plate facing upward, (b) view of plate turned over)

2.4 CONCLUSIONS

Analysis of the substrates used in coal bioconversion studies included elemental and proximate analysis. The South African sub-bituminous coal had a lower carbon content than was expected of this rank of coal, and may be particularly resistant to microbial attack based on its low inherent moisture content. However, a substantial amount of aromatic carbon compounds present in its mobile phase might support microbial growth as a sole carbon source.

A solid media screening assay, including three different sets of indicator compounds, was successfully used to identify fungi with the potential to bioconvert low rank coal. A correlation between the ability of fungi to produce ligninolytic enzymes and to decolourise lignin, humic acid and coal-derived humic acid was observed, and found to be limited to Basidiomycete fungi. Contrary to findings reported elsewhere, the *Trametes* species were able to depolymerise large macromolecules in the absence of an easily utilisable carbon source such as glucose. The utilisation of coal model compounds such as phenanthrenequinone and poly-8-hydroxyquinoline as a carbon source suggested that growth may be supported by similar compounds present in the coal mobile phase, and that mineralisation of such compounds produced from the depolymerisation of low rank coal may occur.

While dye decolourisation and lignin, lignite and humic acid depolymerisation assays using solid media were useful in the detection of ligninolytic and depolymerisation activity, respectively, liquid culture experiments would be necessary to identify specific enzymes potentially involved in coal bioconversion.

T. pubescens was the most active fungal strain in all screening assays and showed the greatest potential to degrade low rank coal. Although *Trichoderma atroviride* ES11 did not appear to produce extracellular ligninolytic enzymes or depolymerise lignin or coal macromolecules, it did have the ability to grow on particulate coal as sole carbon source. The bioconversion of sub-bituminous coal with *T. atroviride* ES11, having been isolated in the Western Cape (SA), and *T. pubescens*, therefore, contributed to the novelty of this research project, and became the focus of work presented in the following chapters.

University of Cape Town

CHAPTER 3: INVESTIGATION OF COAL BIOCONVERSION ACTIVITY OF *TRICHODERMA ATROVIRIDE* ES11 IN SLURRY BIOREACTORS

3.1 INTRODUCTION

In Chapter 2 a solid media screening programme was described that included testing *T. atroviride* ES11, among other fungi, for the ability to produce extracellular ligninolytic enzymes, to depolymerise lignin and coal derived humic acids, and to utilise coal model compounds as a sole carbon source. Although extracellular enzyme activity was not detected and depolymerisation of lignin and coal derived humic acids was not achieved under those specific conditions, *T. atroviride* ES11 showed the ability to utilise lignin, coal derived humic acids, phenanthrenequinone, and poly-8-hydroxyquinoline as a carbon source for growth. Since coal is a solid and is insoluble in most solvents, its degradation in agar plates is constrained by mass transfer limitations, and the yield of biosolubilised product on the plates is not sufficient for chemical characterisation. The objective of work described in this chapter was thus to use slurry reactor systems to determine the extent of biosolubilisation mediated by *T. atroviride* ES11 through qualitative analysis of the biosolubilised product and agents involved in the biosolubilisation process.

T. atroviride ES11, a new environmental isolate, had shown the ability to grow on low rank coal as the sole carbon source (Silva-Stenico *et al.*, 2007). This fungal strain is a member of the deuteromycetes, a group less commonly studied in coal bioconversion compared to the wood-decaying basidiomycetes (see Table 1.3 in Chapter 1). However, the direct isolation of *Trichoderma* spp., and other deuteromycetes from open-cast coal mines, and on decaying wood, has been reported (Laborda *et al.*, 1999; Hölker *et al.*, 1997).

The ability of *T. atroviride* and other *Trichoderma* strains to transform coal has previously been reported by Laborda *et al.* (1999; 1997; 1994) and Hölker *et al.* (2002; 1999; 1997; 1995) working on Spanish and German coals, respectively. The degree to which biosolubilisation was achieved strongly depended upon coal rank and pre-treatment of the coal, with pre-treated lignite having undergone the highest degree of biosolubilisation. A correlation between the nature of the carbon co-substrate and the degree of biosolubilisation was suggested by Hölker *et al.* (1997), and experiments with cell-free culture filtrates implied the involvement of extracellular enzymes such as oxidases and hydrolases, but no conclusive evidence was provided (Hölker *et al.*, 1999).

The work reported here includes the use of slurry systems, specifically shake flask cultures, and the subsequent use of an airlift loop bioreactor, to allow for further monitoring of the process, and products of low rank coal biosolubilisation by the novel wild-type strain, *T. atroviride* ES11. The experiments included investigation of coal biosolubilisation in shaken liquid cultures and resting cell reactions. Bioconversion of coal was determined through the measurement of accumulated low molecular weight aromatic

compounds by chromatographic methods, as well as the common methods described in literature. Enzymatic assays and pH measurements were also performed in order to determine possible agents of biosolubilisation.

The work presented here was carried out in collaboration with E. Silva-Stenico and H. Janjua (Silva-Stenico *et al.*, 2007) at the University of the Western Cape (UWC), and B. Oboirien (Oboirien *et al.*, 2008) at the University of Cape Town (UCT).

3.2 MATERIALS AND METHODS

Reagents were all of analytical or HPLC grade as required, and were obtained from Merck, Fluka or Sigma-Aldrich (RSA). Sub-bituminous coal (SASOL, 2001) was sieved to an average size smaller than 500 µm and was not pre-treated other than sterilisation by autoclaving at 120°C.

3.2.1 Coal bioconversion in shake flask cultures

3.2.1.1 Growth conditions

T. atroviride (ES11) was routinely maintained on 3% malt extract agar at 4°C. The media used for shake flask culture experiments consisted of a minimal salts medium: 1 g $\text{NH}_4(\text{SO}_4)$, 0.52 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 7.6 g KH_2PO_4 , 0.005 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.003 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, supplemented with 0.3% malt extract, 5 g glucose and 10 g sub-bituminous coal per litre. 13 (500 mL) flasks, containing 100 mL medium, were inoculated with 5 agar plugs of active mycelium. 10 glass beads were added to each flask for mycelial disruption. Flasks were incubated at 28°C and 150 rpm. Non-inoculated medium and coal was used as a negative control, while inoculated medium without coal was used as a positive control for growth.

3.2.1.2 Sampling procedure and extraction of products

One flask was harvested daily over a period of 10 days for analysis. Gravimetric analysis was performed by filtering the contents of each flask onto pre-weighed filters (Millipore 0.45 µm) under vacuum. The filters were re-weighed after drying at 80°C overnight. The pH of the supernatant was measured using a Cyberscan 1000 pH meter. The supernatant (20 mL) was extracted with an equal volume of ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulphate. *In vacuo* solvent removal at 45°C was conducted using a Buchi R200 Rotavapor, and the residue was re-dissolved in 2 mL ethyl acetate for HPLC and GC analysis.

In a separate experiment performed as described above, the coal-bound biomass residue was rinsed off the filters with distilled water. The mixture was sonicated using a VirTis Virsonic ultrasonic cell disruptor

100 for 1 minute at maximum power. Samples were centrifuged and the release of any membrane-bound products was measured by HPLC analysis of the supernatant.

3.2.2 Coal bioconversion scaled-up in an airlift loop bioreactor

3.2.2.1 Reactor set-up and culture conditions

A concentric internal draught tube bioreactor with the following specifications was used (Ryan *et al.*, 2005):

Height	500 mm
Aspect ratio (H/D)	4.5
Total working volume	3.5 L
Draught tube diameter	55 mm

An aquarium pump supplied air to the reactor at a fixed rate of 2 L.min⁻¹. The reactor was filled with 3.15 L of growth medium (described in Section 3.2.1) and autoclaved. After cooling, a 10% inoculum was added aseptically and connection to the air supply commenced the experiment.

The pre-inoculum was prepared by aseptically transferring spores using a loop from an agar plate into 200 mL growth medium. The flasks were incubated at 28 °C at 160 rpm for 3 days. The inoculum used for the reactor was prepared in 2 L flasks, containing 400 mL growth medium and 10% seed culture obtained from the pre-inoculum, incubated at 28 °C and 160 rpm.

Since a strong population of *T. atroviride* was obtained overnight in the airlift loop reactor, sterile coal was added on day 2 of growth. Half-strength growth medium was added every 3 days to maintain the working volume.

3.2.2.2 Sampling procedure and extraction of products

The bioreactor was sampled every 24 hours by withdrawing 50 mL of the liquid culture from the sample port on the side of the reactor. The supernatant was obtained by filtering samples onto pre-weighed filters (Millipore 0.45µm) under vacuum. pH, glucose consumption, total phenolic content, and extracellular enzyme activities (laccase, lignin peroxidase, manganese peroxidase) were measured (See section 3.2.4). Discreet off-gas samples, taken every 24 hours, were analysed using a Hertmann & Braun Advanced Optimna Magnos 16 off-gas analyser.

3.2.3 Bioconversion of sub-bituminous coal by resting mycelia of *T. atroviride* ES 11

3.2.3.1 Reaction conditions

T. atroviride ES11 was grown in the medium mentioned above (Section 3.2.1) for 4 days. Mycelia were harvested by centrifugation, using a Beckman Avanti J-25 centrifuge, and transferred to into 100 mL potassium phosphate buffer (pH 5) containing 1.5 g sterile coal. Ammonium sulphate was added as a nitrogen source at a concentration of 1 g L⁻¹. Flasks were incubated at 28°C on an orbital shaker at 170 rpm for ten days.

3.2.3.2 Sampling procedure and extraction of products

The resting mycelia reaction flasks were sampled at 24-hour intervals by removing 2 mL of the reaction liquid. Residual coal and mycelia were removed by centrifugation using a Heraeus Biofuge *pico* benchtop centrifuge. The supernatant was extracted with an equal volume of ethyl acetate and the extract was analysed by gas chromatography.

3.2.4 Analytical procedures

3.2.4.1 Coal biosolubilisation activity – A_{450 nm}

The increase in absorbance of the culture filtrate obtained from the liquid culture of *T. atroviride* with coal, at 450 nm, was measured at 2-day intervals as an indication of the release of humic acids (Cohen *et al.*, 1987). A Helios α Unicam spectrophotometer was used to measure the absorbance at 450 nm. The growth medium was used as a reference blank.

3.2.4.2 Glucose consumption assay

Glucose concentrations were determined using the reducing sugars assay, based on the colourimetric conversion of dinitrosalicylic acid (DNS) according to the Miller (1959). The DNS reagent solution contained 10 g L⁻¹ dinitrosalicylic acid, 2 g L⁻¹ phenol, 0.5 g L⁻¹ sodium sulphite and 10 g L⁻¹ sodium hydroxide. 3 mL of reagent was added to 3 mL sample in a test tube, which was then heated at 90 °C in a water bath for fifteen minutes. This was followed by the addition of 1 mL 40% potassium sodium tartrate solution. The contents of the test tube were allowed to cool to room temperature and the absorbance was read at 575 nm using the Helios α Unicam spectrophotometer. Sample was replaced by distilled water in the reagent blank. A standard curve in the range of 0 – 1 g L⁻¹ was constructed using glucose.

3.2.4.3 Total phenolic content

The total phenolic content was measured using the Folin-Ciocalteu reagent, adapted from the method of Garcia *et al.* (2001). In a 4 mL plastic cuvette, 400 μ L of sample, 400 μ L Folin Ciocalteu reagent and 400 μ L sodium carbonate solution (20%) were added to 2.5 mL distilled water and mixed. After 90 minutes of incubation at room temperature, in the dark, the absorbance was read at 765 nm using the Helios α Unicam spectrophotometer. A standard curve in the range of 0 – 0.1 g L⁻¹ was constructed using gallic acid as a standard and distilled water was used as the reagent blank.

3.2.4.4 Extracellular enzyme activity assays

Laccase activity was measured according to Roy-Arcand and Archibald (1991) by measuring the enzyme-catalysed oxidation of ABTS (2,2' -azinobis 3-ethylbenzthiazolinone-6-sulphonic acid) as the increase in absorbance at 420 nm ($\epsilon = 36\,000\text{ M}^{-1}$). The reaction mixture consisted of 2.5 mL 0.1M sodium acetate buffer (pH 5), 0.33 mL 5 mM ABTS solution and 0.17 mL enzyme sample. 1 unit (U) of enzyme activity was defined as the amount of enzyme required to oxidise 1 μ mole of ABTS per minute.

Manganese peroxidase (MnP) activity was measured according to Castillo *et al.* (1994), based on the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino)benzoic acid (DMAB). In the presence of hydrogen peroxide, Mn²⁺ and MnP, the reaction gives a deep purple-blue colour absorbing at 590 nm ($\epsilon = 53\,000\text{ M}^{-1}\text{cm}^{-1}$). The reaction mixture contained 100 μ L each of MBTH (0.07 mM), DMAB (0.99 mM), MnSO₄ (0.3 mM), H₂O₂ (0.05 mM), succinate buffer (100 mM), lactate buffer (100 mM) and enzyme solution, and 300 μ L distilled water.

Lignin peroxidase was measured according to Tien and Kirk (1988) by measuring the oxidation of veratryl alcohol by H₂O₂ to veratraldehyde as an increase in absorbance at 310 nm ($\epsilon = 9\,300\text{ M}^{-1}\text{cm}^{-1}$). The reaction mixture contained 200 μ L veratryl alcohol (10 mM), 200 μ L sodium tartrate (250 mM), 420 μ L distilled water, 80 μ L H₂O₂ (5 mM) and 100 μ L enzyme solution. Absorbance readings over time were taken for the enzyme assays using the Helios α Unicam spectrophotometer.

3.2.4.5 High performance liquid chromatography (HPLC)

HPLC analysis was performed using the LaChrom System (Merck, Germany) with UV detection (L-7400) using a reverse-phase 5 μ m C18 Waters Spherisorb (250mm x 4.6mm) column. Peaks were detected at 280 nm and analysed using the D-7000 HPLC System Manager. The mobile phase consisted of water/ acetic acid/methanol (77.5: 2.5: 20 v/v/v), and separation was achieved under isocratic conditions.

3.2.4.6 Gas chromatography (GC)

GC analysis was performed on a ThermoFinnigan Focus GC with flame ionisation detection. Nitrogen was used as the carrier gas at a flow rate of 1 mL.min⁻¹, and hydrogen was used as the make-up gas at a flow rate of 40 mL.min⁻¹. An Alpha DexTM 120 capillary column (30 m length x 0.25 mm ID x 0.25 µm film thickness) was used. A 10-µm injection syringe was used. The GC temperature programme is shown in Table 3.1. Peaks were analysed using a Delta 5.5 Chromatography Data System.

Table 3.1 GC temperature programme

		Temperature (°C)	Time (min)
Oven	Initial	135	5
	Ramp	10	Per minute
	Final	220	40
Injector		225	-
FID		250	-

3.3 RESULTS AND DISCUSSION

3.3.1 Coal biosolubilisation by *T. atroviride* in shake flasks

In order to evaluate coal biosolubilisation activity of *T. atroviride* ES11 in liquid cultures, submerged flasks containing basal medium supplemented with malt extract, glucose and sub-bituminous coal were inoculated and incubated for 10 days. Coal biosolubilisation was measured by an increase in absorbance at 450 nm and by the release of low molecular weight intermediates, measured chromatographically or chemically using the total phenolic assay. The pH of the culture filtrate was monitored to determine whether a correlation between change in pH and biosolubilisation existed.

Humic acids were released as products of coal biosolubilisation during the liquid culture of *T. atroviride* ES11 with sub-bituminous coal, as indicated by the broad linear increase in $A_{450\text{ nm}}$, shown in Figure 3.1. Cohen *et al.* (1987) stated that this was the simplest qualitative means of monitoring coal solubilisation because background absorbance, caused by the culture itself, was negligible at this wavelength. However, an increase in $A_{450\text{ nm}}$ was observed in the culture grown in the absence of coal, although this only occurred until the fourth day; thereafter, a plateau in the absorbance was observed. No humic acids were released due to chemical leaching reactions or physical attrition, based on the very small changes in $A_{450\text{ nm}}$ observed in the abiotic control (coal and medium, in the absence of fungus).

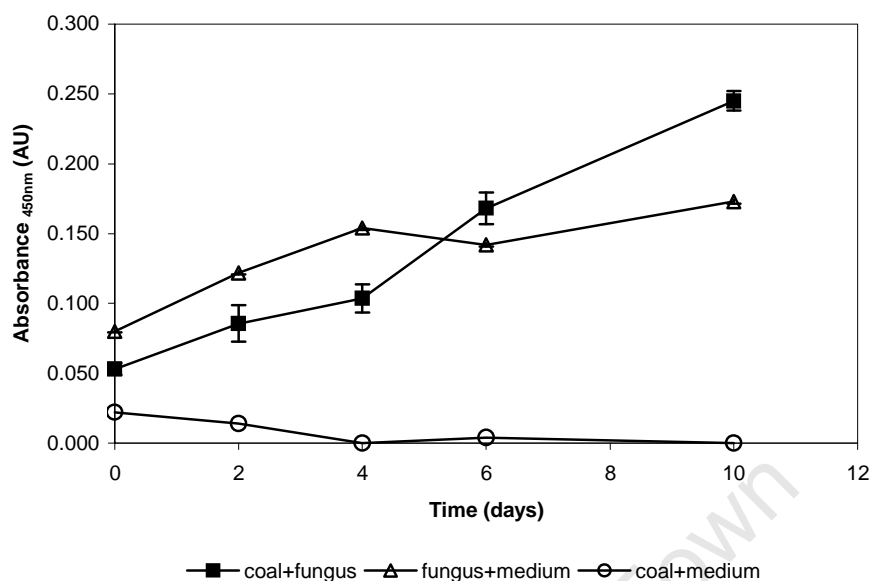


Figure 3.1 Increase in absorbance at 450 nm as a measure of the release of humic acid resulting from coal biosolubilisation by *T. atroviride* ES11

Monitoring the increase in $A_{450\text{ nm}}$ as a measure of coal biosolubilisation was, until recently, the most frequently used method. Laborda *et al.* (1997, 1999) used this method to measure the biosolubilisation of lignite, sub-bituminous coal and hard coal by environmental isolates, *Trichoderma* sp. M2 and *Penicillium* sp. M4. According to their results, both strains were effective in biosolubilising all coals, but the degree of biosolubilisation was dependent on the type of carbon source used. Lignite was biosolubilised when strains M2 and M4 were grown in a minimal medium containing an easily utilisable carbon source, such as glucose, while hard coal and bituminous coal was only solubilised when a complete medium, containing an organic nitrogen source, was used (Laborda *et al.*, 1997). However, a satisfactory correlation between the increase in $A_{450\text{ nm}}$ and gravimetric analysis of residual coal was not achieved, and inoculated medium without coal was not used as a control. In subsequent experiments reported by this group, lignite was treated with nitric acid to facilitate its biosolubilisation by strain M2 and M4 (Laborda *et al.*, 1999). In most experiments on coal biosolubilisation reported in literature, the coal was pre-treated with acid or hydrogen peroxide, leading to an increase in its water-solubility due to the increase in the oxygen content, and removal of multivalent cations which form bridges contributing to the structural stability of coal. The increase in $A_{450\text{ nm}}$ attributed to the increase in water-solubility, due to pre-treatment of coals, should be accounted for when presenting coal biosolubilisation activities, by ensuring that the relevant abiotic control experiments are performed. Since the data from relevant controls were not always presented, the degree of coal biosolubilisation is questionable.

Biosolubilisation of lignite by *T. atroviride* and *Fusarium oxosporium* was also measured as the increase in $A_{450\text{ nm}}$ by Hölker *et al.* (2002, 1999, 1997 1995), but again, culture controls were not mentioned. These soil fungi are also known to produce melanins – darkly-coloured, high molecular weight pigments, which have certain similarities to humic acids e.g. UV and visible absorption spectra, and precipitation by acid (Zavgorodnyaya *et al.*, 2002; Butler and Day, 1998). The proposed function of these melanins is to provide protection against lytic enzymes, desiccation and irradiation. Melanins can be detected at $A_{465\text{ nm}}$, and this means that monitoring a change in $A_{450\text{ nm}}$ is not a conclusive method for determining coal biosolubilisation, and should only be used in conjunction with other methods, such as gravimetric analysis of coal residues or chromatographic measurement of products.

In the present study, in order to overcome the limitation associated with spectrophotometric measurements of coal biosolubilisation, HPLC analysis of culture filtrates, along with the appropriate controls, from the shake flask experiments with *T. atroviride* ES11 and coal was performed, and chromatograms are shown in Figure 3.2. Similar profiles were obtained for culture filtrates regardless of the absence or presence of coal. The presence of UV-active compounds, produced by *T. atroviride* ES11 in the absence of coal, varied throughout the culture growth period, and the production and/or accumulation of UV-active intermediate compounds specifically from the biosolubilisation of coal was not observed. A reasonable explanation for this might be the adsorption of intermediates to the fungal mycelia, or that no UV-active compounds were produced. It should also be note that humic acids would not elute from the C18 column under the conditions used due to its large size.

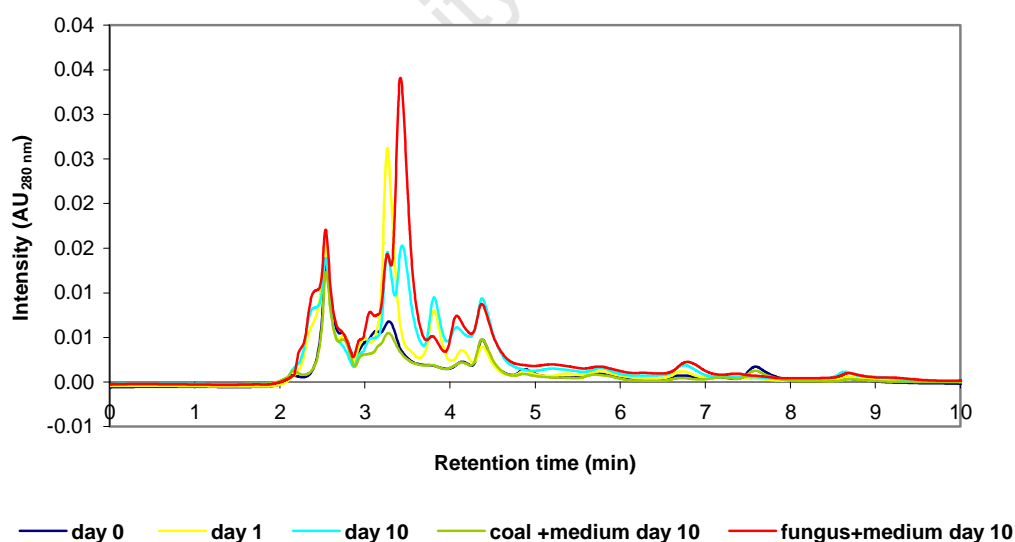


Figure 3.2 HPLC chromatogram showing the time-related changes in elution profile during the growth of *T. atroviride* ES11 in the presence and absence of coal

Ralph and Catcheside (1996) demonstrated the binding of alkali-solubilised brown coal to the mycelia of white rot and brown rot fungi. The bound solubilised coal was recovered by alkali washing of the mycelia. The adsorption of organic compounds to mycelia during the degradation of aromatic compounds by white rot fungi, causing an apparent increase in the percentage degradation of the aromatic substrate, has also been previously reported, and this effect was addressed by extracting the fungal biomass with organic solvent and/or homogenisation of the fungal biomass in an organic solvent (Mi-Sun *et al.*, 1998; Dhawale *et al.*, 1992).

In this study the effect of sonication on the removal of adsorbed organic compounds, produced from coal biosolubilisation by *T. atroviride* ES11, from the mycelia, was tested. The release of these compounds was measured by HPLC and the chromatograms are displayed in Figure 3.3. The majority of peaks eluted between 2 and 5 mins, similar to Figure 3.2. However, additional peaks were also eluted after 5 mins, suggesting that those compounds were more non-polar in nature. The compounds released from the fungal mycelia after sonication also appeared with higher intensity than in the abiotic control, containing coal and medium in the absence of fungus. The release of UV-active compounds from fungal mycelium grown in the absence of coal, and non-inoculated medium and coal, was negligible, suggesting that the UV-active compounds were products of coal biosolubilisation. However, these compounds were bound to the mycelia of *T. atroviride* ES11 and detection by HPLC was only possible if sonication was applied to assist in the release of these compounds. This was a key finding because it confirmed that aromatic organic compounds, which are possibly products of coal biosolubilisation by *T. atroviride* ES11, were bound to the mycelia, and since accumulation of these compounds was not observed, it was possible that absorption may have facilitated further metabolism of the compounds to form biomass.

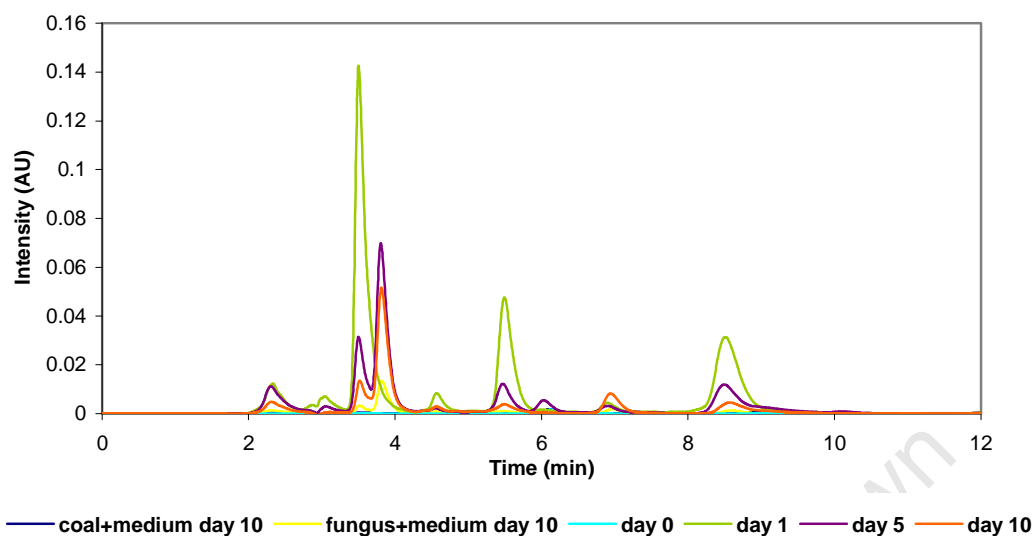


Figure 3.3 HPLC chromatogram showing the effects of sonication on the release of compounds bound to fungal hyphae during the growth of *T. atroviride* ES11 in the presence and absence of coal

The production of volatile organic intermediate compounds, which are not necessarily UV-active, from the biosolubilisation of coal was measured by gas chromatography (GC). The GC chromatogram of ethyl acetate extracts of the culture filtrate from the liquid culture of *T. atroviride* ES11 in the presence and absence of coal is shown in Figure 3.4. An increase in intensity (or concentration) and number of volatile organic compounds occurred as the growth of *T. atroviride* ES11 proceeded, in the presence of coal.

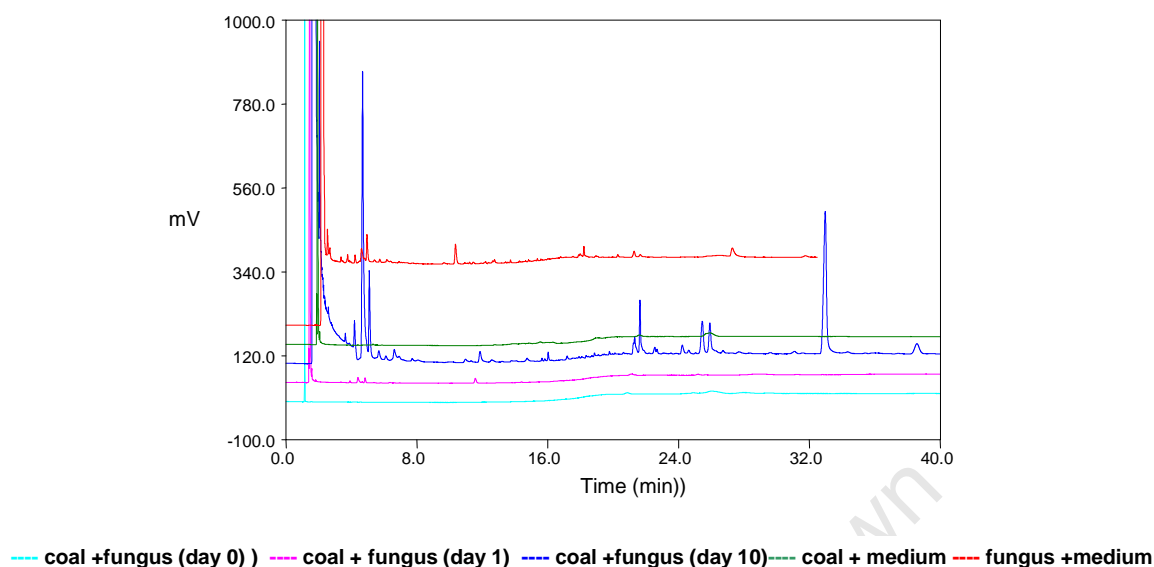


Figure 3.4 GC chromatogram showing the increase in concentration and number of volatile compounds produced during the growth of *T. atroviride* ES 11 with coal over time

In collaborative work carried out in this project, gas chromatography – mass spectroscopy (GC-MS) analysis of culture filtrates, obtained after an 11-day culture of *T. atroviride* ES11 with coal, extracted with chloroform and silylated with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), allowed identification of 3 compounds; 4-hydroxyphenylethanol, 2-octenoic acid and 1, 2-benzenediol (chemical structures in Figure 3.5) (Silva-Stenico *et al.*, 2007). To our knowledge, this is only the second report naming specific products of coal biosolubilisation, and is the first report to name products of coal biosolubilisation by a *T. atroviride* strain.

4-Hydroxyphenylethanol (commonly known as tyrosol), octenoic acid and 1,2 benzendiol (catechol) belong to structural classes that are expected from coal depolymerisation and secondary oxidative reactions. Hofrichter *et al.* (1997) reported the production of 2- hydroxybiphenyl, alkylated benzenes (e.g. *o*-diethylbenzene) and polyaromatic hydrocarbons (e.g. fluorene) from powdered hard coal by *Coprinus sclerotigenis* C142-1, a litter-decaying fungus. The mechanism by which these compounds were liberated could not confidently be stated, but an enzymatic attack was presumed, although activities of oxidative enzymes were not detectable and the effect of hydrolytic enzymes had not yet been investigated (Hofrichter *et al.*, 1997).

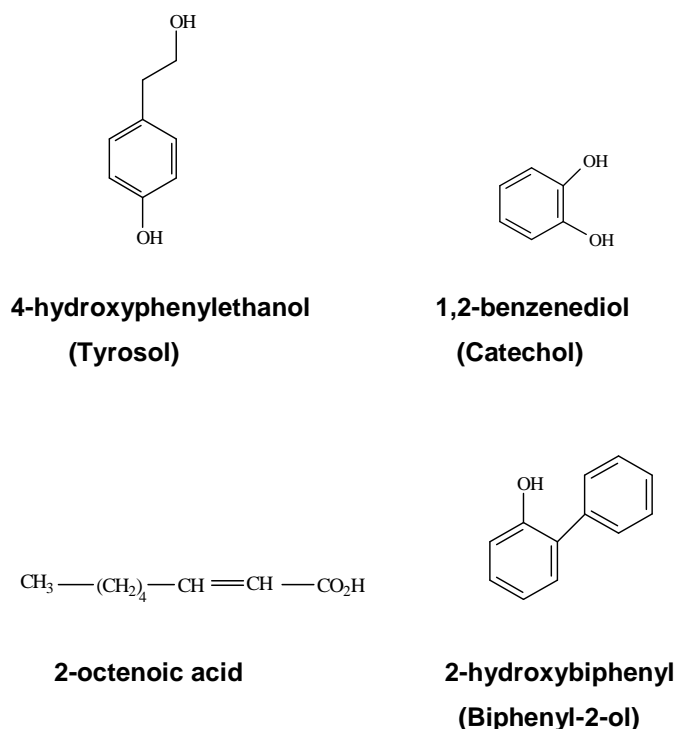


Figure 3.5 Structures of compounds found to be products of coal biosolubilisation (Silva-Stenico *et al.*, 2007; Hofrichter *et al.*, 1997)

Although three compounds were identified as products of coal biosolubilisation in this study, the limited diversity of aromatic compounds detected is questionable owing to the fact that previous reports on the biosolubilisation of coal described the product to be a mixture of polar organic compounds, ranging in molecular mass and having a high degree of aromaticity (Scott *et al.*, 1986). The physical association of coal particles and fungal mycelium, together with the adhesion of coal-derived intermediates to fungal mycelium (as described above) possibly facilitates the metabolism of these intermediates into biomass and carbon dioxide.

The ability of ES11 to utilise simple aromatic model compounds such as phenanthrenequinone, as carbon source, was described in Chapter 2. Thus, the degradation of the aromatic structure, followed by uptake of the products by the hyphae, must have occurred in order to support fungal growth. Furthermore, collaborative work with Oboirien *et al.* (2008) following from experiments in this study, showed that soluble products from the biosolubilisation of coal by *T. atroviride* ES11 were further degraded to form biomass and carbon dioxide. The experiments were conducted in shake flask cultures using the same coal and growth medium as used in the present study. The measurement of total organic carbon content of the supernatant was used to define the soluble components in the carbon balance. In addition to this, coal

biosolubilisation was measured gravimetrically (coal weight loss) and spectrophotometrically at 450 nm, and by performing a simple carbon balance, the authors deduced that further metabolism of soluble intermediates resulted in the oxidation of a considerable portion of coal to carbon dioxide.

In the present study, gravimetric analysis of residual coal after biosolubilisation was found to be a tedious method of determining the degree of coal biosolubilisation and representative samples could not be taken from culture fluids because fungal hyphae became attached to coal particles. Germinating fungal hyphae had a strong affinity towards coal particles, and the effect appeared to increase as the hyphae aged, as was also described by Fakoussa (1988). It was therefore very difficult to construct an effective mass balance over the system since the net weight of a sample (containing biomass and residual coal) might not change: a decrease in coal weight due to biosolubilisation may result in an increase in biomass weight. This uncertainty is illustrated in Figure 3.6 which shows the change in dry mass of biomass, and biomass/coal residue (which could not be physically separated) during the growth of *T. atroviride* ES11 with coal. In this experiment, multiple flasks were used, where the whole content of each flask represented a 24-hour sample, and therefore only one data set is presented here. The conventional way of carrying out the experiment in triplicate flasks, where smaller volumes of sample would be daily removed from the same flask, was not performed, because a greater sampling inaccuracy was associated with this method due to the non-homogeneous nature of the contents of the flask, which resulted from the binding of fungal hyphae to coal particles.

Hypothetically, if coal was not degraded initially, one would be able to measure an increase in dry weight of biomass/coal residue due to the formation of new biomass on the existing glucose, as is seen in the line representing biomass in the absence of coal. Conversely, a decrease in dry weight of the biomass/coal residue was observed until day 2, followed by a slight increase and then a levelling out of dry weight until day 5. Subsequently, an increase was observed until day 8, followed by a decrease. Although the dry weight of the coal/biomass residue varied over time, the overall net weight was maintained over the 10-day period, indicating that the products of coal biosolubilisation by *T. atroviride* ES11 may have been assimilated to form more biomass.

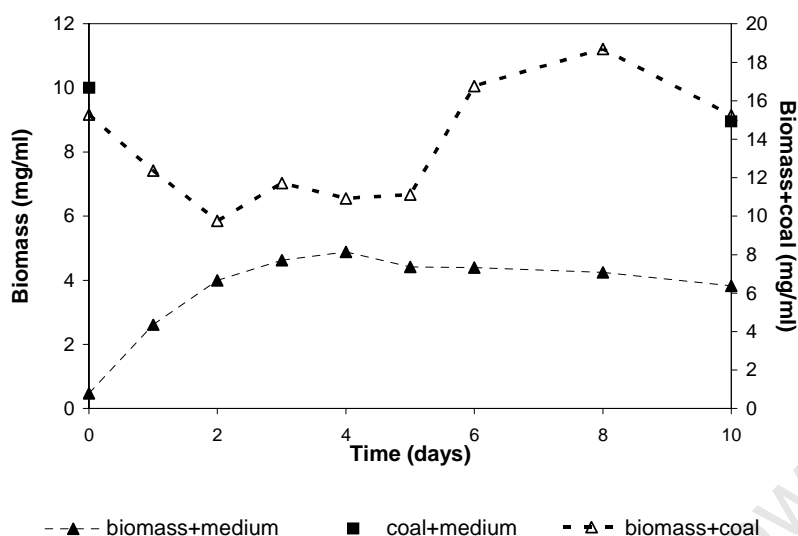


Figure 3.6 Weight of biomass (biomass yield), biomass and coal residue measured over a 10-day period during growth of *T. atroviride* ES11 (Sampling of flasks containing coal and medium alone were only taken at the start and end of the experiment due to risk of contamination.)

The pH of the filtrate obtained from the culture of *T. atroviride* ES11 grown with coal in shake flasks, was measured and is depicted in Figure 3.7. A decrease in pH from 5.69 to 3.4 was noted in filtrates from cultures grown both in the presence and absence of coal. The pH of non-inoculated medium with coal did not change. This observation excludes the suggestion that alkali production might be a mechanism for coal bioconversion by *T. atroviride* ES11. This was an important result, and together with the increase in $A_{450\text{ nm}}$, HPLC and GC analysis of the culture filtrates, confirmed that humic acids and other products were produced from the bioconversion of coal by *T. atroviride* ES11 by a different agent, either produced extracellularly or cell-wall associated, and not as a result from the solubilising effect of an alkaline supernatant.

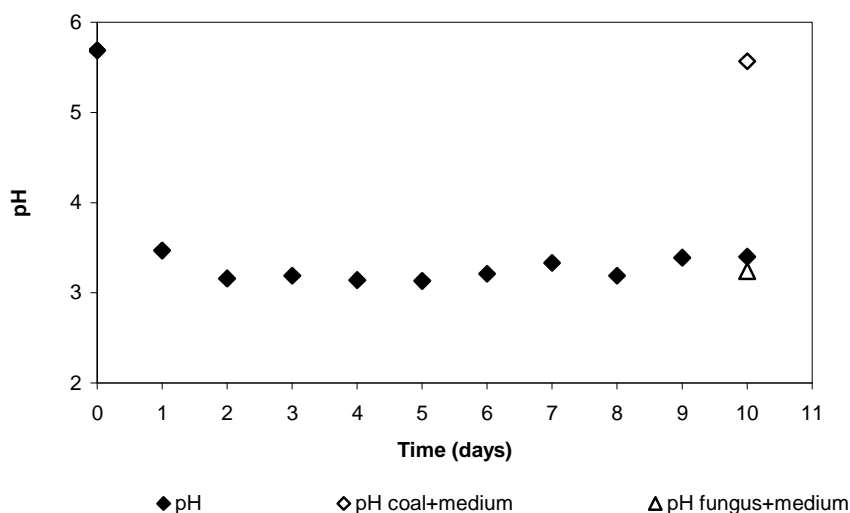


Figure 3.7 pH variation over time during growth of *T. atroviride* ES11 with coal in shake flasks (Sampling of flasks containing coal and medium alone were only taken at the start and end of the experiment due to risk of contamination.)

Hölker *et al.* (1999) showed that when *T. atroviride* and *F. oxosporium* were grown on carboxylic carbon sources (e.g. glutamate or gluconate), solubilisation of pre-treated lignite occurred as the pH increased to 9, but when the fungi were grown on carbohydrate carbon sources (e.g. glucose or maltose), the pH decreased to 3 and biosolubilisation did not occur. The increase in pH occurred as a result of the presence of free bases, formed from the remaining mono and dibasic metals (Na^+ , K^+ , Ca^{2+}) which remain after the utilisation of organic acids, such as sodium gluconate (Hofrichter and Fakoussa 2001). Thus, alkaline supernatants of fungal mycelia displayed much stronger solubilising effects on lignite than aqueous buffer solutions of equal pH (Hölker *et al.*, 1999). Contrary to the findings of Hölker *et al.* (1999), biosolubilisation of sub-bituminous coal by *T. atroviride* ES 11 occurred even though a decrease in pH was observed, re-confirming that alkali production is not a mechanism used by *T. atroviride* ES11 to biosolubilise coal.

The ultimate aim of this study was to produce lower molecular weight aromatic compounds from the bioconversion of coal. The proposed way to achieve this would be through extracellular enzymatic cleavage of the coal matrix, during which production of humic acids might be the initial step, followed by its degradation to yield smaller aromatic compounds. Glucose was therefore used as the carbon source for growth of *T. atroviride* ES11 during the bioconversion of sub-bituminous coal, based on the results reported by Hölker *et al.* (1999), that decolourisation of coal-derived humic acids and the simultaneous production of extracellular laccase only occurred when *T. atroviride* was grown in media containing

glucose. Investigation of the extracellular enzyme activities of *T. atroviride* ES11, namely laccase (lac), manganese peroxidase (MnP) and lignin peroxidase, (LiP), during the bioconversion of sub-bituminous coal was performed using colorimetric assays. None of these enzymes were detected in the liquid cultures, corroborating the results obtained during the solid media screening assays based on the decolourisation of Poly R-478, described in Chapter 2. However, supernatants extracted from *T. atroviride* ES11 cultures grown in the presence of coal, were able to reduce the coal dry weight by 10%, while no reduction was observed when autoclaved supernatants were used (Silva-Stenico *et al.*, 2007). Thus the possible involvement of extracellular enzymes, although not necessarily those mentioned above, should not be overlooked.

In summarising the results described above, it was evident that *T. atroviride* ES11 degraded sub-bituminous coal in shake flasks, based on the variation in the weight of biomass/coal residue, production of humic acids, and the production of tyrosol, octenoic acid and catechol. However, the decrease in pH and inability to produce ligninolytic enzymes suggested that biosolubilisation did not occur through the production of alkaline substances or production of ligninolytic enzymes. A study of the intracellular enzyme activity of *T. atroviride* ES11 during the bioconversion of sub-bituminous coal, conducted by collaborators, showed that several dehydrogenase enzymes, which are typically involved in the degradation pathways acting on polycyclic aromatic hydrocarbons (PAH) in bacteria, were induced in the presence of coal (Silva-Stenico *et al.*, 2007). These included 2,3-dihydrobiphenyl-2,3-diol dehydrogenase; 3,4-dihydrophenanthrene-3,4-diol dehydrogenase; 1,2-dihydro-1,2-dihydroxy-naphthalene dehydrogenase and 1,2-dihydro-1,2-dihydroxyanthracene dehydrogenase. Catechol 1,2-dioxygenase activity was also detected but was decreased in the presence of coal (Silva-Stenico *et al.*, 2007). This is interesting because the degradation pathway of PAH compounds is initiated by the conversion of the substrates to *cis*-dihydrodiols by catechol dioxygenases, and the reaction is followed by re-aromatisation and ring cleavage catalysed by a number of dehydrogenases, yielding smaller compounds more suitable for further metabolic processing as nutrient carbon (Muller, 1992). The close association of fungal hyphae and coal particles, coupled with the detected intracellular enzyme activities mentioned above, and the release of products bound to hyphae (upon sonication) suggested that the sub-bituminous coal was degraded by *T. atroviride* ES11, and that the resulting products were catabolised, supporting further growth of the fungus.

It should also be noted that non-ligninolytic fungi typically use monooxygenases, such as cytochrome P450-monoxygenases, to oxidize and activate aromatic compounds (Harayama, 1997). Thus, future work should also include an investigation of monooxygenase activity of *T. atroviride* ES11 during coal bioconversion.

3.3.2 Coal bioconversion by *T. atroviride* ES11 in an airlift loop reactor

In a further investigation of the effect of *T. atroviride* ES11 on the biosolubilisation of coal, shake flask culture experiments were scaled up in an internal loop airlift reactor to afford a greater working volume for analyses. Glucose content, total phenolic content and off-gas analysis, monitored through the measurement of discrete samples, was performed. A photograph of the bioreactor is shown in Figure 3.8.

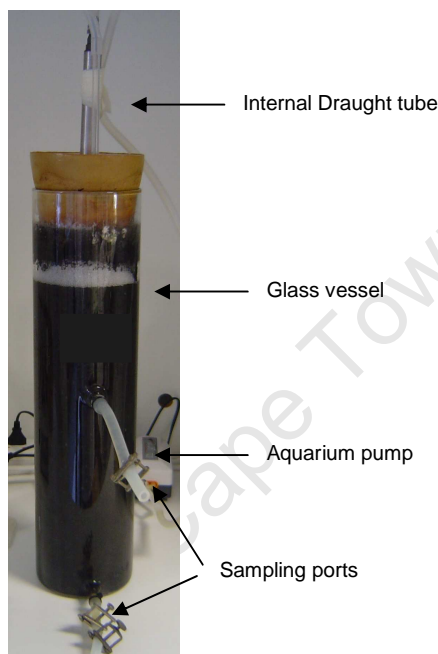


Figure 3.8 Photograph of the 3.5 L airlift loop bioreactor used in the bioconversion of sub-bituminous coal by *T. atroviride* ES11

Growth of *T. atroviride* ES11 in the airlift reactor occurred more rapidly than in shake flask cultures, probably due to the increase in oxygenation achieved through the introduction of greater volumes of air to the reactor per unit time. The addition of sterile coal to the reactor was administered after glucose depletion which occurred by day 2, in order to encourage the utilisation of coal carbon by *T. atroviride* ES11. A half-strength growth medium was added on day 3, 5 and 8 to maintain the working volume of the reactor, and to ensure that the system did not become nitrogen-limited. The half-strength medium also provided glucose, an easily metabolisable carbon source, that was shown to be necessary for the solubilisation of lignite by fungal strains isolated from coal (Laborda *et al.*, 1997). Although small amounts of glucose were added to the reactor, a negligible increase in glucose concentration was observed using the reducing sugars assay, indicating that the added glucose was consumed within a day.

Gravimetric analysis of biomass and coal samples taken from the airlift bioreactor showed an initial increase in dry weight of the combined biomass and coal residue, followed by an overall decrease (Figure 3.9). This was in contrast to the initial decrease followed by an overall increase, obtained in the shake flask culture experiments where coal was added at the time of inoculation.

While the addition of the half-strength medium to the airlift bioreactor made it difficult to quantitatively analyse the bioconversion of coal by *T. atroviride* ES11, an interesting relationship between biomass/coal dry weight and carbon dioxide concentration was noted, particularly between day 5 and 8. Thus, a decrease in dry weight seemed to correspond approximately with an increase in carbon dioxide concentration, which suggested that a decreasing dry weight was due to the solubilisation of coal and that simultaneous mineralisation of the products resulted in an increase in CO₂ released. The CO₂ concentration on day 9 was above 520 ppm, exceeding the level measurable by the analyser at the time. At this stage of growth, the reactor became overgrown with biomass and wall growth began to block circulation in the reactor, and the experiment was ended on day 9.

The total phenolic content of the supernatant samples taken from the airlift bioreactor was measured, showing an initial decrease until day 3 of culture growth, followed by plateau in concentration until the end of the experiment. No increases in total phenolic content were observed suggesting that no phenolic compounds were released during the bioconversion of coal by *T. atroviride* ES11 in the airlift bioreactor. A collaborative investigation (carried out by colleagues in our laboratory) into the effect of coal particle size and solids loading on the biosolubilisation of sub-bituminous coal by *T. atroviride* ES11 was conducted (Oboirien *et al.*, 2008). Here, a considerable increase in total organic carbon (TOC) content of the supernatant, from an initial 330 mg L⁻¹ to over 1000 mg L⁻¹, was observed 2 days after the addition of coal, at a 10% (w/v) coal loading and 600 - 850 µm coal particle size, to shake flask cultures of *T. atroviride* ES11. This was followed by a rapid decrease in TOC and a residual amount of approximately 300 mg L⁻¹ was maintained thereafter (Oboirien *et al.*, 2008). This evidence suggested that, following the release of soluble organic compounds from coal, these compounds are further mineralised to carbon dioxide. Collectively, the results from the TOC analysis (in the study by Oboirien *et al.*, 2008) and the corresponding increase in CO₂ concentrations, correlating with a decreasing dry weight of biomass/coal samples observed in the present study, led to the conclusion that sub-bituminous coal was degraded by *T. atroviride* ES11 to form biomass and CO₂, and confirmed the results obtained in shake flask culture experiments.

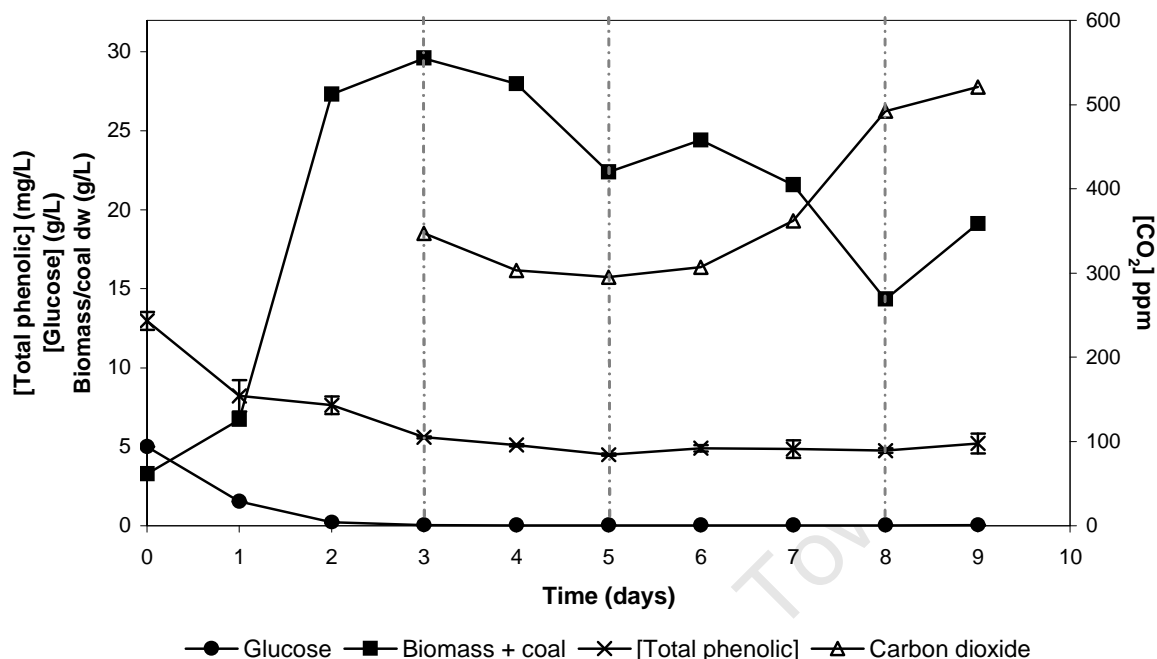


Figure 3.9 Measurement of glucose, total phenolics and carbon dioxide concentrations taken during growth of *T. atroviride* ES11 with coal in the ALR (The dashed lines indicate days on which half-strength basal medium was added to the reactor to maintain the working volume. Sterile coal was added on day 2.)

Although submerged cultures are commonly used in industry for the production of enzymes, they are not without limitations, e.g. uncontrolled growth of fungal mycelium that can result in mass transfer limitations and blocking. As an alternative, filamentous fungi can be cultured by solid substrate fermentation (SSF), particularly since they have an inclination to adhere to surfaces and to grow whilst immobilised on their substrates in nature (Couto and Toca-Herrera, 2007). Hölker and Höfer (2002) investigated SSF of lignite by *T. atroviride* to obtain useful amounts of solubilised product for chemical analysis. Culturing was carried out in a semi-continuous, fed-batch mode in the presence of glutamate. The degree of solubilisation was monitored photometrically at 450 nm and residual coal was measured gravimetrically at the end of the culturing time. 9.3% coal was biosolubilised over 40 days even though the reactor became contaminated after 20 days and by day 40, *T. atroviride* could no longer be detected. No mention was made of the mechanism of solubilisation but an increase in pH to values above 7 was observed, suggesting that biosolubilisation due to the production of alkaline substances had occurred. Although these reports indicate that SSF is a promising route for the application of coal biosolubilisation, SSF can be problematic in terms of control of pH, temperature, moisture and oxygen transfer (Hölker *et al.*, 2004; Couto and Toca-Herrera, 2007), and scale-up for industrial purposes poses even greater challenges. Clearly, careful design and optimisation, with particular consideration of the substrate characteristics and growth

behaviour of microorganisms, is still required in order to provide an optimised process for the successful bioconversion of coal to yield value added products.

3.3.3 Bioconversion of sub-bituminous coal by resting mycelia of *T. atroviride* ES11

In this study, it was proposed that the metabolism of coal biosolubilisation products may be minimised if mycelia were not actively growing, but were rather only undergoing maintenance reactions. This hypothesis was tested by monitoring the reaction of *T. atroviride* ES11 resting culture with sub-bituminous coal. Thus, *T. atroviride* ES11 was cultivated on the basal medium containing glucose (described in section 3.2.1) and then transferred into phosphate buffer at pH 5, containing sterile coal (15 g L⁻¹) and ammonium sulphate (1g L⁻¹) as a nitrogen source. A pH of 5 was chosen for the resting culture reactions because this was the starting pH of medium used to grow *T. atroviride* ES11 in liquid cultures with coal. The reaction was allowed to proceed for ten days at 28°C with shaking at 170 rpm.

GC analysis of the ethyl acetate-extracted supernatant from the resting cell reactions with coal indicated that *T. atroviride* ES11 resting cells were not capable of bioconverting sub-bituminous coal since no new products were detected and the colour of the supernatant remained clear. This result confirmed the necessity for an additional carbon source (and hence supported the concept of co-metabolism as discussed in Section 2.3.2.2) and, possibly, the extracellular fluid which may contain extracellular agents involved in initial coal breakdown. However, numerous peaks were observed in the abiotic control samples where no fungus was present, suggesting the presence of volatile compounds in the mobile phase of sub-bituminous coal, and confirming the results of the volatile content of coal obtained from the proximate analysis (described in Chapter 2). A decrease in the number and intensity of these peaks were observed in the test reactions containing both coal and fungus, indicating that *T. atroviride* ES11 utilised the compounds present in the mobile phase of sub-bituminous coal (Figure 3.10).

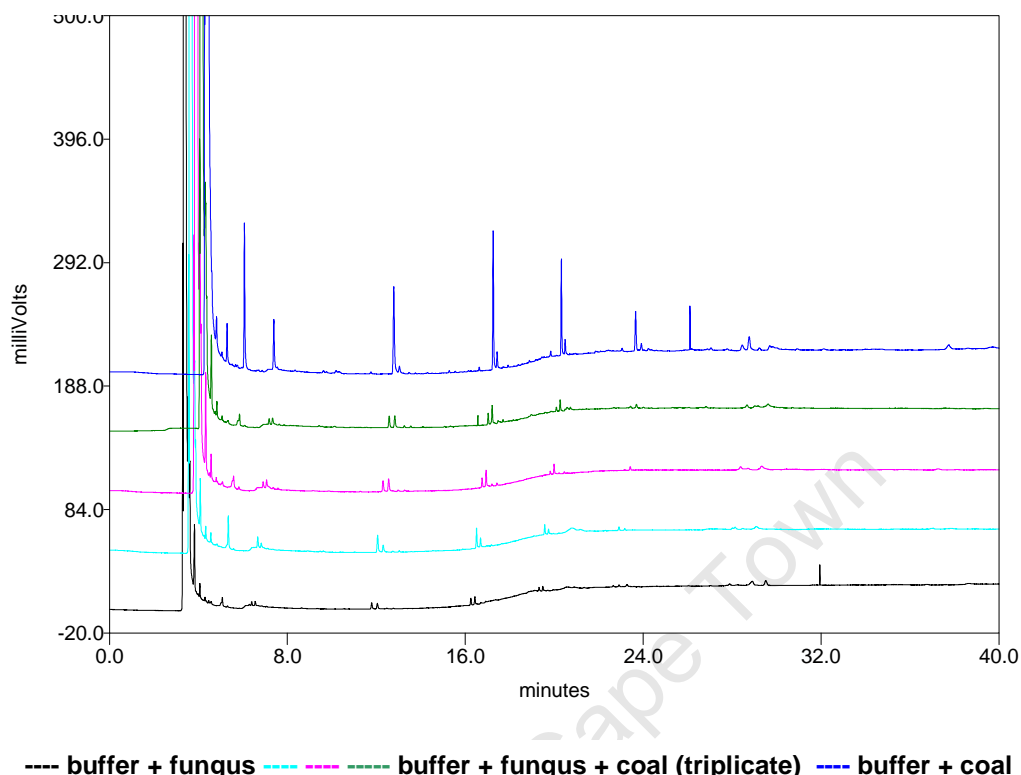


Figure 3.10 GC chromatogram showing the utilisation of compounds, by the resting mycelia of *T. atroviride* ES11 in phosphate buffer (pH 5), which were present in higher concentrations in the abiotic control (containing phosphate buffer and coal) than in the reaction mixture containing resting mycelia and coal.

Most reported investigations of coal conversion have involved liquid cultures or the use of cell-free filtrates. However, resting cell reactions have been described by Bublitz *et al.* (1994) and Ralph and Catcheside (1993), investigating the degradation of asphaltene and alkali-solubilised tetrahydrofuran-insoluble lignite as the sole carbon source, respectively. Bublitz *et al.* (1994) reported that a *Trichoderma* sp. displayed the ability to alter the chemical structure of asphaltene, the product of hard-coal hydrogenation, by increasing the proportion of carbonyl groups and reducing ether linkages, but changes in the molecular weight spectrum (indicating the ability to depolymerise coal macromolecules) of the residual asphaltene, dissolved in an organic solvent, were not detected. The authors did not provide any suggestions with respect to the mechanisms by which the *Trichoderma* sp. was able to alter the chemical nature of asphaltene. However, the increase the proportion of carbonyl groups and reduction in ether linkages could possibly be attributed to the action of esterases or oxidases, which were shown to be involved in the biosolubilisation of lignite by a *T. atroviride* strain (Hölker *et al.*, 2002).

Ralph and Catcheside (1993) showed that isolates able to grow on the alkali-solubilised tetrahydrofuran-insoluble lignite were capable of utilising the aliphatic side chains which are attached to the macromolecular matrix of coal, without breakdown of the coal matrix itself. Similarly, in the current study, *T. atroviride* ES11 resting mycelia showed the ability to utilise volatile compounds present in the coal mobile phase but were unable to break down the coal macromolecular structure. These findings are in agreement with the reports described above, although the substrates and test microorganisms were different. It was proposed that *T. atroviride* ES11 metabolised intermediate products from the bioconversion of coal and that the metabolism of these products may be minimised if mycelia were not actively growing, but were rather only undergoing maintenance reactions. However, the results obtained in this study confirmed that *T. atroviride* ES11 was unable to breakdown the coal macromolecule to yield smaller aromatic compounds, and the presence of the fungal metabolites, produced in liquid cultures, was necessary for coal bioconversion to occur.

3.4 CONCLUSIONS

The work described in this chapter was conducted with the objective of demonstrating the capability of *T. atroviride* ES11 to bioconvert untreated, sub-bituminous coal in slurry reactor systems. An approach to understanding the process was made by monitoring coal bioconversion in liquid cultures (containing particulate coal, and glucose as an additional carbon source), and resting mycelium reactions (in potassium phosphate buffer, with coal as the sole carbon source).

Spectrophotometric analysis at 450 nm of the supernatants from liquid cultures with coal suggested the release of humic acids resulting from the action of *T. atroviride* ES11 on coal. However, compounds produced by the fungus, in the absence of coal, also absorbed at this wavelength, necessitating alternative analyses that could provide data to substantiate coal bioconversion. Gravimetric analysis of the residual coal would theoretically be an ideal method of quantifying coal bioconversion, but, as is common in systems involving filamentous microorganisms, the fungal mycelia became tightly bound to the coal particles. Coal bioconversion could also be determined through measurement of the release of UV-active, volatile organic compounds by HPLC or GC; here HPLC analysis of the filtrate obtained after the sonication of fungal mycelia, removed from the liquid culture of *T. atroviride* ES11 with coal, showed the presence of more products than were measured in the supernatant from the liquid culture itself. This confirmed the attachment of coal bioconversion products to fungal mycelia.

An increase in the production of volatile organic compounds was observed during growth of ES11 with sub-bituminous coal in shake flasks, measured by GC, and in collaborative studies, three products of the coal biosolubilisation were identified as 4-hydroxyphenyl ethanol (tyrosol), 1,2-benzenediol (catechol) and

2-octenoic acid. These compounds have valuable applications and suggested that the biological processing of sub-bituminous coal could be useful.

The bioconversion of sub-bituminous coal by *T. atroviride* ES11 and further metabolism of products, to yield biomass and CO₂, was facilitated by the close interaction between coal and fungal hyphae. Since products of coal bioconversion were mineralised by actively growing *T. atroviride* ES11 mycelia, the investigation of the bioconversion of sub-bituminous coal by resting mycelia of *T. atroviride* ES11, to minimise mineralisation reactions and allow the accumulation of desirable low molecular mass intermediates, was investigated. However, the bioconversion of coal was found to require actively growing mycelia and/or its extracellular products and therefore the presence of an additional carbon source was required.

The general conclusions drawn from work described in this chapter were, therefore, that *T. atroviride* ES11 degraded sub-bituminous coal to form humic acids which were further degraded to lower molecular mass compounds such as tyrosol, catechol and 2-octenoic acid. However, the product yield was very low because these compounds were further metabolised to form more biomass and carbon dioxide. Genetic manipulation of the fungus may be necessary in order to block pathways involved in the metabolism of primary coal catabolites and redirect the carbon-flow to improved product yields. This would not be a trivial task and would require a thorough understanding of the metabolic pathways involved in coal bioconversion by *T. atroviride* ES11.

An extracellular mechanism of coal degradation is required due to the insolubility of coal and size of its macromolecules, and since *T. atroviride* ES11 did not produce extracellular ligninolytic compounds or alkaline substances, the mechanism by which ES11 initially attacks coal remains unclear. It is possible that extracellular hydrolytic enzymes such as esterases were produced. Research in this regard was not pursued, based on the fact that esterases act in a specific manner (i.e. on ester linkages), and therefore, products from the bioconversion of coal obtained in this way would not be as diverse as those obtained through the action ligninolytic enzymes, which are known to act in a non-specific manner. The action of esterases on high molecular mass coal macromolecules may also be limited due to steric fit, and esterases are not known to act via mediator molecules, such as those produced by ligninolytic enzymes, which may act directly on bonds within the coal matrix. The basidiomycete strains, *T. versicolor* and *T. pubescens* were able to produce ligninolytic enzymes, as discussed in Chapter 2, and thus, further research into coal bioconversion with these strains became the focus of subsequent experiments.

CHAPTER 4: COAL BIOCONVERSION ACTIVITY OF *TRAMETES* SPP.

4.1 INTRODUCTION

The objective of the work reported in this chapter was to investigate the degradation of low rank coal and related compounds by *Trametes versicolor* and *Trametes pubescens* in liquid culture. The bioconversion of coal by *T. pubescens*, having shown noteworthy potential based on the solid media screening assay described in Chapter 2, contributed a degree of novelty to this research project. Although the biosolubilisation of low rank coal, particularly lignite, by *T. versicolor* has been extensively researched and is well-documented, it was selected as a benchmark strain in the present study on the bioconversion of sub-bituminous coal. Further, following from the results of work on *Trichoderma atroviride* ES11 (which showed that it could bioconvert sub-bituminous coal, but further metabolism of soluble intermediates occurred resulting in production of biomass and carbon dioxide), ligninolytic activity (as demonstrated by these *Trametes* species) seemed to be necessary to achieve the ultimate objective of producing low molecular mass intermediates through the depolymerisation of low rank coal. The bioconversion of sub-bituminous coal, lignite and asphaltene by *Trametes* spp. was therefore investigated.

4.2.1 Substrates used in coal bioconversion studies

The background information on sub-bituminous coal and lignite (such as chemical structure and susceptibility towards microbial attack) was discussed in Chapter 1 (see Sections 1.2.3 and 1.2.4). In this section, asphaltene was also investigated because it is a hydrogenation product of hard coal that is soluble in organic solvents, and is regarded a basic structural unit of hard coal (Bublitz, *et al.*, 1994; Hofrichter *et al.*, 1997). Asphaltenes are also present in the wax-free fraction of petroleum which is insoluble in *n*-heptane but soluble in hot toluene/benzene (Almehaideb, 2004). It should be noted that there are structural differences between petroleum-derived and coal-derived asphaltenes, which is largely dependent on the source material, as well as the methods and conditions used to obtain the asphaltenes (Mullins and Sheu, 1998). Extensive studies on the structure of asphaltenes have shown that they contain condensed aromatic systems carrying alkyl, cycloalkyl and heteroatom constituents (Figure 4.1) (Trejo *et al.*, 2004). Asphaltene was used as a suitable model compound to test for the ability of fungi to degrade hard coal (Hofrichter *et al.*, 1997), based on the fact that asphaltene does not have a mobile phase containing low molecular, readily volatilized compounds, and therefore, detection of new compounds would be due to enzymatic attack as opposed to leaching of substances. Further, the presence of asphaltenes in crude oils is problematic due to the formation of deposits near the wellbore, and they are also known to be coke-precursors, causing deactivation of the catalyst during heavy oil hydrotreating. Thus, a method for removal (or partial removal) of this high molecular weight fraction of oil would be highly

beneficial and of great economic value to the petroleum industry, which provides an additional incentive for investigating its breakdown by white rot fungi.

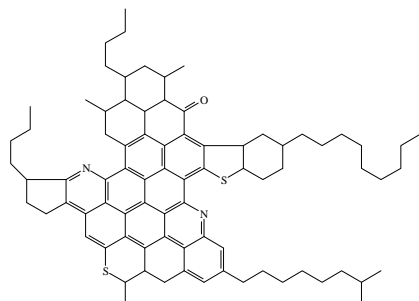


Figure 4.1 Model structure of petroleum-derived asphaltene (Speight, 1992)

4.2.2 Strains and enzymes used in the bioconversion of coal

T. versicolor and *T. pubescens* belong to the Basidiomycotina family, which cause white rot in wood. These fungi are commonly found on dead logs, stumps, tree trunks and branches (Cui and Christi, 2003). Based on this, white rot fungi (WRF) have been extensively studied and shown to be efficient degraders of lignin (Goodell *et al.*, 1997). The lignin-degrading systems of white-rot fungi are extracellular (and include oxidase enzymes). Thus, the fungi can often degrade insoluble chemicals such as hazardous environmental pollutants. The non-specific nature of the mechanisms used by these enzyme systems allows them to degrade even complex mixtures of pollutants. Initiation of degradation can often be achieved by limiting the nutrient source or through the addition of certain aromatic compounds, which induces the production of oxidative enzymes (Barr and Aust, 1994; Kantelinen *et al.*, 1989).

The ability of WRF to produce oxidative enzymes (oxidases) has been extended to numerous other applications such as the degradation of recalcitrant organo-pollutants and industrial dyes (Dec and Bollag, 1990; Morgan *et al.*, 1991); and more recently, coal biodegradation. Lignite and leonardite (naturally weathered lignite) coal have been the most commonly investigated substrates in coal biodegradation. A number of mechanisms for the bioconversion of lignite by *T. versicolor* have been proposed, but they are not definitive and in many cases are contradictory (Torzilli and Isbister, 1994). *Trametes* species are known to be one of the main producers of the ligninolytic enzyme, laccase (Jang *et al.*, 2002; Galhaup *et al.*, 2002). There exist numerous contradictory reports on the effect of laccase on coal biodegradation and biosolubilisation (yielding coal macromolecules in solution), discussed in detail below.

Cohen and Gabriele (1982) first reported the direct growth of *T. versicolor* and *Poria monticola* on lignite particles. At that stage, not much was known about the degradation of lignin by white rot fungi but since

lignite coals originate from plant material and lignin-like structures are preserved during the coalification process, an understanding the WRF lignin degradation processes could be applied to the biosolubilisation of lignite. Wilson *et al.* (1987) also demonstrated that partially purified enzyme preparations from *T. versicolor* could solubilise leonardite but Cohen *et al.* (1987) showed that the addition of exogenous laccase did not have an observable effect of the rate of coal biosolubilisation. In a study done by Pyne *et al.* (1987), cyanide and azide were shown to inhibit cell-free biosolubilisation of leonardite by laccase extracted from *T. versicolor*.

The first identified coal solubilising agent (CSA) produced by *T. versicolor* was found to be ammonium oxalate monohydrate (Cohen *et al.*, 1990). The crystal structure was confirmed by x-ray studies. Conversely, Fredrickson *et al.* (1990) found that *T. versicolor* produced a siderophore-like product which, through complexation of iron and other metal cations, acting as bridges between organic moieties, resulted in biosolubilisation. The possible action of enzymes was negated since heating at 100 °C for forty minutes had no effect on coal biosolubilisation.

Later, Torzilli and Isbister (1994) revisited the nature of the CSA produced by *T. versicolor*. They found that solubilisation of coal was caused by increased alkalinity, above pH 8. Separation of CSAs by Sephadex G-50 showed products of low molecular mass. This, and the thermostable nature of the culture filtrate, precluded an enzymatic mechanism of coal biosolubilisation. The addition of potassium oxalate had a greater effect on coal biosolubilisation than ammonium oxalate, and when ammonium ions were removed, an increase in solubilisation was achieved. This suggested that the solubilising activity lay with the oxalate moiety and that ammonium ions were possibly inhibitory.

In contrast to solubilisation attributable to chemical interactions, reduction of the molecular weight of coal substances, resulting from the breakage of carbon-carbon bonds (depolymerisation) was suggested to only be achievable biologically, in the presence of extracellular, non-specific and stable enzymes (Fakoussa and Hofrichter, 1999). Further analyses of catalytic mechanisms were subsequently investigated, including exploration of different nutrient conditions that favour coal biodegradation. In lignin biodegradation studies, different nutrient conditions resulted in different enzyme activities and lignin degradation capabilities (Ralph *et al.*, 1996). For example, *T. versicolor* produces a large amount of laccase, depending on media constituents, culture conditions and culture age. The depolymerisation of coal-derived humic acids by *T. versicolor* occurred while the fungus was producing large amounts of laccase (Fakoussa and Frost, 1999). However, the presence of whole coal particles seemed to decrease the measurable laccase activity, suggesting a sensitivity of laccase to the lignite particles.

A new strain, *Trametes pubescens* (CBS 696.94), recently isolated from an ash-tree in Austria, was found to be an outstanding producer of extracellular laccase (Galhaup and Haltrich, 2001). Apart from the

characterisation of this new species and its enzymes (Galhaup *et al.*, 2002; Maresova, *et al.*, 2005; Shleev *et al.*, 2007), other research foci have included the use of *T. pubescens* in the degradation of phenolic wastewaters (Ryan, 2003), decolourisation of synthetic dyes in solid-state fermentations (Osma *et al.*, 2006, 2007) and biotransformations of phenolic compounds to produce antioxidants using the isolated laccase (Nicotra *et al.*, 2004; Ncanana and Burton, 2007). This study reports, for the first time, the biodegradation of sub-bituminous coal by *T. pubescens*.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

Reagents were all of analytical or HPLC grade as required, and were obtained from Merck, Fluka or Sigma-Aldrich chemical companies (RSA), unless otherwise stated. 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was purchased from Roche (Germany). Sub-bituminous coal (SASOL, 2001) was sieved to an average size smaller than 500 µm and was not pre-treated other than sterilisation by autoclaving at 120°C.

4.2.2 Strain preservation, inoculation and growth conditions

Trametes versicolor (PPRI # 3845) and *Trametes pubescens* (CBS 696.94) were obtained from the Plant Protection Research Institute (Pretoria, RSA) and the Boku Institute in Austria, respectively. The strains were routinely subcultured on 3% malt extract agar and stored at 4°C.

Trametes Defined Medium (TDM) (Addleman and Archibald, 1993. See Appendix A) was used for liquid culture experiments and included veratryl alcohol (24.4 µM), which was used to induce laccase activity produced by *Trametes* spp. The inoculum was prepared by homogenising plate cultures in pre-autoclaved growth medium. Static and shake flask cultures were prepared in Erlenmeyer flasks containing TDM at a growth medium (including 10% inoculum) to air ratio of 1:4. The flasks were incubated at 28°C.

4.2.3 Coal bioconversion by *T. versicolor* in static cultures

Static cultures of *T. versicolor* were prepared as described in Section 4.2.2. Triplicate flasks were incubated statically at 28°C for 7 days *i.e.* until the culture reached mid-log growth phase, visible by the formation of a thick fungal mat. 15 g sterile coal was added to the surface of the fungal mat. After 3 weeks, the culture fluid was carefully decanted and retained for enzyme activity assays. The biosolubilised coal was removed and dissolved in an equal volume of ethyl acetate (polarity index = 4.4). The products were not soluble in ethyl acetate and therefore, an equal volume of methanol (polarity index = 5.1) was added. The mixture was then filtered under vacuum using a Buchner funnel to remove the solid coal residue. *In*

vacuo solvent removal was carried out using a Buchi R200 Rotavapor to concentrate the filtrate, which was set aside for HPLC analysis.

4.2.4 Coal bioconversion in shake flask cultures

4.2.4.1 Shake flask cultures of *T. versicolor* with low carbon nutrient content and coal added at the time of inoculation

Coal bioconversion activity of *T. versicolor* was monitored in shaken (submerged) flask cultures. The same growth conditions were used as described above for static cultures (Section 4.2.3), except that after inoculation, the flasks were incubated at 28°C on a n orbital shaker at 150 rpm. The glucose concentration was also decreased by half and a quarter of that normally present in TDM (normal carbon content 1%). This experiment was performed in triplicate, with non-inoculated medium containing coal used as a negative control, and inoculated medium without coal was used as a positive control for growth. 15 g sterile sub-bituminous coal was added to each flask at the time of fungal inoculation. After 4 weeks of incubation, residual coal and fungal hyphae were removed by vacuum filtration. Laccase activity determinations, UV-VIS spectrophotometric analysis and HPLC analysis were performed on the aqueous fraction and organic extracts of the culture filtrates.

4.2.4.2 Shake flask cultures of *T. versicolor* and *T. pubescens* with coal added after 5 days of growth

At this stage in the project, an alternate strain of *Trametes*, *Trametes pubescens* (CBS 696.94) was acquired. *T. versicolor* and *T. pubescens* were grown in 100 mL TDM in 500 mL flasks. 1 g of sterile sub-bituminous coal was added to shake flask cultures after 5 days of growth, coinciding with the first day of sampling. All flasks were incubated at 28°C and agitated at 180 rpm. Samples were collected every 2 days for two weeks and stored at -20°C until the end of the experiment. Biomass and residual coal were separated from the culture filtrate by vacuum filtration. The residue was weighed and treated with nitric acid (acid digestion) to remove fungal hyphae. Microanalysis of the remaining coal was performed to determine the C, H, N, O and S content of the remaining coal. The filtrate was analysed by UV-VIS spectrophotometry and HPLC. Laccase enzyme activity was also measured using the ABTS assay as described in Section 4.2.6.1.

Filtrates from the submerged *T. pubescens* cultures (collected on the last day of incubation) were extracted using ethyl acetate. Aqueous and organic phases were analysed by reverse phase HPLC. The coal-bound hyphal residue was treated by alkali-washing and homogenisation in an attempt to remove the residual coal. All the analytical procedures are described in Section 4.2.6.

4.2.4.3 Shake flask cultures of *T. versicolor* with lignite added after 7 days

This experiment was performed in a 2-L shake flask to enable more frequent sampling without removing more than the accepted 10% of the total volume. 50 g of lignite coal was added to a seven day old population of *Trametes versicolor* cells grown in 1.2 L of TDM. The flask was aerated using an aquarium pump at a flow rate of approximately 2 L.min⁻¹ and incubated at 28°C on an orbital shaker at 150 rpm. Samples were taken daily for the first week and subsequently every 2-3 days. Analyses included laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) activity, and UV spectrophotometry, as described in Section 4.2.6.

4.2.5 Bioconversion of asphaltene by *T. pubescens* in submerged cultures

A 5-day old culture (prepared as described in Section 4.2.4.1) of *T. pubescens* was homogenised aseptically. 1 mg asphaltene was added to 1 mL homogenate in each of 16 screw-cap vials. The negative control consisted of asphaltene and medium, whilst the positive control contained homogenate and medium. 10 µL Tween 80 solution (1%) was added to each vial to aid in submersion of asphaltene in the medium. The vials were placed horizontally on a gel- rocker at 28°C. One vial was sacrificed daily over a period of 14 days, each representing a 24-hour sample. Vials were centrifuged at 13000 rpm for 5 minutes to separate cells and asphaltene from the supernatant. 1 mL tetrahydrofuran (THF) was added to the remaining pellet to recover the residual asphaltene. The vials were centrifuged again, the THF-dissolved asphaltene was pipetted into clean vials and the THF was allowed to evaporate. The asphaltene particles, remaining after bioconversion with *T. pubescens*, were visualised using scanning electron microscopy (described in Section 4.2.6.9).

The experiment was repeated in 20 mL conical flasks using a reaction volume of 5 mL, under the same reaction conditions as above. The flasks were incubated at 28°C on an orbital shaker at 130rpm. Cells and asphaltene were filtered from the supernatant after 3 weeks of incubation. The remaining asphaltene and attached fungal hyphae was dried and weighed. The supernatant was retained for HPLC analysis (see Section 4.2.6.3).

4.2.6 Analytical procedures

4.2.6.1 Enzyme assays

Laccase activity was determined by following the oxidation of 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Wolfenden and Willson, 1982). 0.17 mL of filtrate was added to 2.5 mL sodium acetate buffer (0.1M, pH 5) and 0.33 mL of the ABTS solution (5 mM) in a 3 mL cuvette (Roy-Arcand and Archibald, 1991). The change in absorbance was read at 420 nm over time using a Unicam Helios Alpha/Beta UV-Vis spectrophotometer. An extinction coefficient of 36 000M⁻¹.cm⁻¹ (Roy-Arcand and

Archibald, 1991) was used to determine laccase activity, where 1 U of activity is equivalent to the amount of laccase required to catalyse the conversion of 1 μmol of substrate per minute.

Manganese peroxidase activity was measured according to Del Pilar Castillo *et al.* (1994) following the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-dimethylaminobenzoic acid (DMAB). In a 1 mL cuvette, 100 μL of the culture filtrate was added to 900 μL of reagent mixture. The reagent mixture consisted of sodium succinate buffer (100mM, pH 4.5); sodium lactate buffer (100 mM, pH 4.5); MBTH (0.99 mM); DMAB (0.07 mM); MnSO_4 (0.3 mM), H_2O_2 (0.05 mM) and distilled water. The cuvette was inverted to mix, and the absorbance was read at 590 nm on the Unicam HeLios Alpha/Beta UV-Vis spectrophotometer, over time. An extinction coefficient of $53\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ (Del Pilar Castillo *et al.*, 1994) was used to determine MnP activity, where 1 U of activity is equivalent to the amount of MnP required to catalyse the conversion of 1 μmol of substrate per minute.

Lignin peroxidase was measured according to Tien and Kirk (1988) by measuring the oxidation of veratryl alcohol by H_2O_2 to veratraldehyde as an increase in absorbance at 310 nm ($\epsilon = 9\,300\text{ M}^{-1}\cdot\text{cm}^{-1}$). The reaction mixture contained 200 μL veratryl alcohol (10 mM), 200 μL sodium tartrate (250 mM), 420 μL distilled water, 80 μL H_2O_2 (5 mM) and 100 μL enzyme solution. 1 U of activity was equivalent to the amount of LiP required to catalyse the conversion of 1 μmol of substrate per minute.

4.2.6.2 Liquid-liquid organic extraction

The culture filtrate was extracted three times with equivalent volumes of methyl ethyl ketone or ethyl acetate. The organic fractions were pooled and evaporated to dryness on a Buchi R200 Rotavapor. The residue was re-dissolved in a minimal amount of HPLC mobile phase (see below) or distilled water. These samples were then analysed immediately or stored at 4°C until required.

4.2.6.3 High performance liquid chromatography (HPLC)

HPLC analysis was performed using a LaChrom System (Merck, Germany) with UV detection (L-7400). Samples were separated on a reverse-phase 5 μm C18 Waters Spherisorb (250mm x 4.6mm) column. Peaks were detected at 280 nm and analysed using a D-7000 HPLC System Manager. Initially, the mobile phase consisted of water, acetic acid, methanol (79: 1: 20 v/v/v) but later, more acetic acid was added to give concentrations of 77.5: 2.5: 20 v/v/v (water/acetic acid/methanol).

4.2.6.4 Gas chromatography

A Perkin Elmer Autosystem GC chromatograph fitted with a SGE Sol-Gel WAXTM column (Polyethylene glycol in Sol-Gel matrix, 30 m length x 0.32 mm ID x 0.5 μm film thickness) was used. The GC trace integration was carried out with Delta 5.5 chromatography software using a DataworX DataCentre 4000

interface board. 1 μ L sample was analysed under the following conditions: nitrogen as carrier gas, temperature programming 60°C (5 min), 60-200°C (29 min), 200 °C (30min), 200-250°C (40min), 250 °C (45 min).

4.2.6.5 UV-VIS spectrophotometric analysis

Samples were thawed and centrifuged at 13 000 rpm for 3 minutes. 1 mL of the supernatant was added to a quartz cuvette. A spectral scan was taken over a wavelength range of 800 nm to 200 nm with 20 nm intervals using the Shimadzu UV-160 spectrophotometer. Coal biosolubilisation was also monitored by the increase in absorbance at 450 nm and 360 nm, indicating the formation of humic and fulvic acids, respectively (Cohen *et al.*, 1987; Fakoussa and Frost, 1999).

4.2.6.6 Alkali-washing, homogenisation and light microscopy analysis of coal-bound mycelia

Coal-bound mycelia obtained from submerged culture reactions with *T. pubescens*, were treated with 3M NaOH overnight to remove the coal from the fungal hyphae (method adapted from Ralph and Catchside, 1997). This mixture was later homogenised with 100 mL distilled water, filtered and dried. Fixative and lactophenol blue was added to a small amount of the residue and examined under 4x magnification using a light microscope, to determine the degree of coal removal. Fungal cell debris stained blue, while coal appeared black.

4.2.6.7 Acid digestion

The coal and biomass residue, obtained after separation from the spent culture medium (see Section 4.2.4.2), was weighed into 250 mL Erlenmeyer flasks. 20 mL Nitric acid (60%) was added to the flasks, which were then placed on a heating block (> 100 °C) in a fumehood. A flask containing 1 g of native sub-bituminous coal and 20 mL nitric acid solution was used as a control. The mixture boiled until 5 mL of liquid remained. The reaction between the coal and nitric acid gave off a brown NO₂ vapour. The remaining liquid was filtered on pre-weighed Whatman paper. The residue-containing filters were dried in an oven at 80°C overnight and then re-weighed.

4.2.6.8 Microanalysis

Microanalysis of coal was conducted by the Department of Chemistry, University of Cape Town. The method employs a Thermoflash 1112 series C, H, N, S and O analyser with combustion analysis in pure oxygen and GC column separation using helium gas. Compounds are detected using differential thermal conductivity.

4.2.6.9 Scanning electron microscopy

The asphaltene particles were fixed onto a slide using graphite and glue. This procedure was performed in the Electron Microscopy Unit in the Physical Science Department at the University of Cape Town.

4.3 RESULTS AND DISCUSSION

The objective of the work presented here was to determine the coal bioconversion capabilities of *T. versicolor* and *T. pubescens* in liquid culture. Sub-bituminous coal, lignite and asphaltene, representing coals of different rank, were used as substrates, with the aim of gaining some understanding of the coal biodegradation process.

4.3.1 Bioconversion of sub-bituminous coal by *T. versicolor* in static flasks

T. versicolor was grown in static cultures until a thick mycelial mat developed on the surface of the growth medium. Sterile sub-bituminous coal was added to the top of the mat to keep the coal particles separate from the growth medium, and the culture was further incubated until solubilisation of the coal occurred. Biosolubilisation of coal, evident from 4-5 days after its addition, was evidenced by the conversion of powdered coal to a liquid form above the fungal mat (Figure 4.2). The growth medium below the mycelial mat remained yellow in colour. In some cases, biosolubilisation was not visible but growth of hyphae on the solid coal particles was evident.



Figure 4.2 Biosolubilised coal on the surface of *T. versicolor* hyphal mat

The flasks were incubated for 3 weeks after which the supernatant was decanted and the partially biosolubilised coal was removed by pipetting the liquid and scrapping the residual coal from the fungal mat, using a spatula. An attempt was made to extract the products into an organic solvent, viz. ethyl

acetate, but extraction of the coloured products into the organic phase did not occur. After removal of the ethyl acetate layer, methanol was added to the aqueous mixture. The residual coal particles were removed by filtration, and the biosolubilised coal, mixed in the methanol was concentrated, producing a yellow residue; this was subjected to reverse-phase separation by HPLC. Untreated coal was also extracted using methanol and this solution was analysed as a control. The results are depicted in Figure 4.3. The HPLC profile of the biosolubilised coal was observed to be markedly different from that of the control. The compounds represented by each peak are likely to be polar compounds, since they dissolved in methanol. A few small peaks were observed in the biosolubilised coal extract (indicated by arrows in Figure 4.3) that were not present in the control, suggesting the formation of new products. Furthermore, most peaks present in the control sample (peaks 1, 3, 4, 5 and 6) were no longer detectable in the biosolubilised extract, or a decrease in concentration as evidenced by a decrease in peak area (e.g. peak 2), had occurred. This suggested that these compounds had been utilised by the fungus or that biosolubilisation had caused structural changes to the chromophores that confer absorption in the UV range.

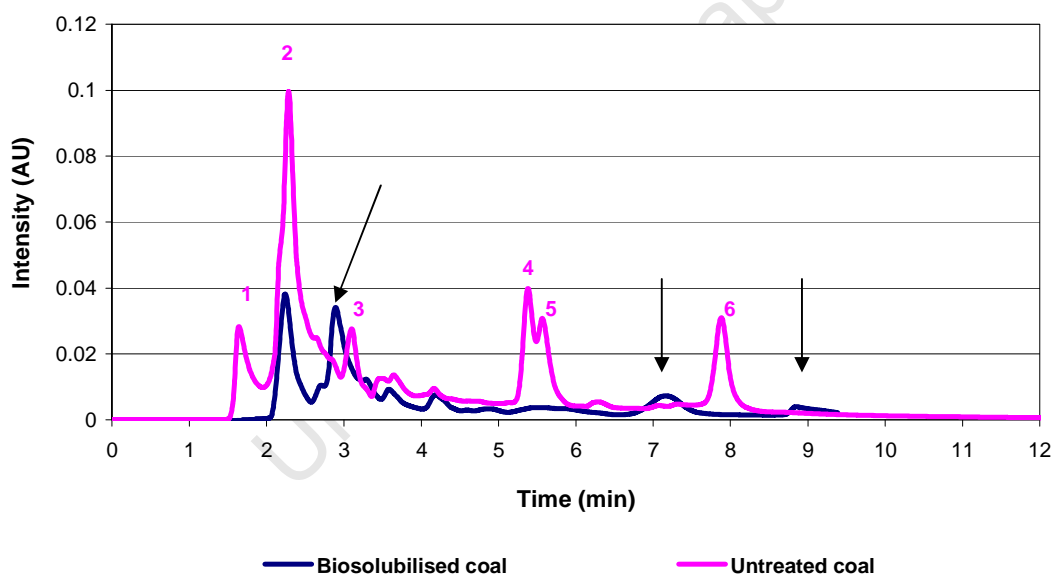


Figure 4.3 HPLC chromatogram of methanol extracts of biosolubilised and untreated coal. The mobile phase consisted of water, acetic acid and methanol (79:1:20). The arrows indicate possible products of coal biosolubilisation which are not present in the control sample. The peaks labelled 1-6 indicate compounds present in the control sample which were not observed in the biosolubilised coal extract.

At this stage of experimentation, comment could not be made on the mechanism by which *T. versicolor* degrades coal. Sampling of the growth medium beneath the mycelial mat was not possible during the

culturing time since coal particles may have been displaced. However, laccase activity, detected in the supernatant on the last day of culturing, was 0.42 U.mL^{-1} as compared with the control, 0.38 U.mL^{-1} , which was a slight difference. No LiP was detectable in this strain of *T. versicolor* and a very low titre of MnP was measured (see section 4.3.2 Figure 4.9).

Although *T. versicolor* produces all three extracellular enzymes typically involved in lignin biodegradation (laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP)), there is wide variability in their occurrence under different culture conditions. In many white rot fungi, ligninolytic enzyme production only occurs under nutrient limitation, but in *T. versicolor*, high levels of laccase may be produced under conditions of rapid (nutrient sufficient) growth. In order to follow the time-related production of ligninolytic enzymes, and to monitor the effects of the presence of coal during its bioconversion by *T. versicolor*, experiments in shake flask cultures were conducted.

4.3.2 Coal bioconversion by *Trametes* spp. in shake flasks

According to Hölker *et al.* (1997), coal biosolubilisation is induced following contact between fungus and coal, and furthermore, the extracellular oxidative enzymes produced by fungi are responsible for the degradation of coal (Willmann and Fakoussa, 1997). Oxidase enzymes, such as laccase, require molecular oxygen as a co-substrate (Bourbonnais and Paice, 1990). Based on this information and the results obtained from the solid media screening (described in Chapter 2) and static flask culture experiments with *T. versicolor* (described above), experiments were performed in shake flask cultures.

4.3.2.1 Shake flask cultures of *T. versicolor* with low carbon nutrient content and coal added at the time of inoculation

T. versicolor was grown in shake flasks containing Trametes Defined Medium (TDM) in which the normal glucose concentration of 1% was reduced to 0.25% and 0.5%. Powdered sub-bituminous coal (15 g.L^{-1}) was added at the same time as inoculation. The glucose content was reduced to minimise the time it would taken to be depleted, thereby increasing the affinity of *T. versicolor* for coal as a carbon source and therefore its coal biodegradative capacity. After 4 weeks of incubation the laccase activity was measured and was found to be higher in cultures containing coal than in control cultures without coal (Table 4.1). This suggested a possible inductive effect. Laccases may have been inducible by compounds which are mainly phenolic in nature, and are structurally related to lignin and its derivatives (Gianfreda *et al.*, 1999). It is therefore possible that this inductive effect was achieved through the release of phenolic compounds from the bioconversion of coal. The *T. versicolor* strain used in the current study has been shown to produce both inducible and growth-associated laccase (Ryan, 2003), and therefore the higher laccase activity produced in cultures grown in 0.5 % glucose was possibly attributable to higher biomass production.

Table 4.1 Laccase activity of *T. versicolor* shake flask cultures with reduced glucose content, grown in the presence and absence of sub-bituminous coal

Glucose concentration	Laccase activity (U. mL ⁻¹)*	
	With coal	Without coal
0.25%	0.092	0.001
0.5%	0.430	0.109

* Mean standard deviation of $\pm 20\%$

Typically, the biosolubilisation of lignite coal is followed by the production of humic acids which can be measured spectrophotometrically at 450nm (Cohen *et al.*, 1987; Fredrickson *et al.*, 1990; Torzilli and Isbister, 1994; Temp *et al.*, 1999; Gokcay *et al.*, 2001). The depolymerisation (C-C cleavage) of low rank coal or coal-derived humic acids can be measured by following the formation of lower-molecular-mass fulvic acid-like compounds (Hofrichter *et al.*, 1999) at 360 nm. In the present study, the absorbance of the filtrates from shake flask cultures of *T. versicolor* with sub-bituminous coal, at 450 nm, was considerably higher than that of the controls where no coal was present and where no fungus was present. A higher absorbance at 360 nm was also measured in cultures containing coal, although the difference between these and the absorbance of filtrates from control flasks was lower (Table 4.2).

Table 4.2 Spectrophotometric data obtained from *T. versicolor* shake flask cultures grown in the presence and absence of sub-bituminous coal (with 0.25% glucose in TDM)

Wavelength	Absorbance (AU)*		
	Test ^a	Control ^b	Control ^c
450 nm	0.745	0.016	0.094
360 nm	0.765	0.276	0.274

^a *T. versicolor* + coal ^b *T. versicolor* + growth medium ^c Sub-bituminous coal + growth medium in absence of fungus *Mean standard deviation of $\pm 25\text{--}35\%$

The spectral scan, from 200 nm to 800 nm, of the supernatant obtained from *T. versicolor* shake flask cultures showed absorbance maxima between 350 and 450 nm for cultures grown in the presence of coal, as opposed to the absorbance maxima obtained between 250 and 350 nm for the controls (Figure 4.4). It is likely that humic acids and fulvic acids were formed during the bioconversion of sub-bituminous coal by *T. versicolor*, based on these results. There are many reports on the production of humic and fulvic acids resulting from the bioconversion of lignite by white-rot fungi. However, only a few reports on the

bioconversion of sub-bituminous coals exist, and in these, the coals were pre-treated with nitric acid to make them more amenable to degradation by fungi and bacteria (Scott *et al.*, 1986; Laborda *et al.*, 1999; Machnikowska *et al.*, 2002). Microbial solubilisation of coal is greatly enhanced through the pre-treatment with various oxidants (Scott *et al.*, 1986), but this is not desirable for industrial bioprocessing applications. Thus, the results presented here are promising, since no pre-treatment was used, and hence, quantitative analysis of the reaction products was warranted.

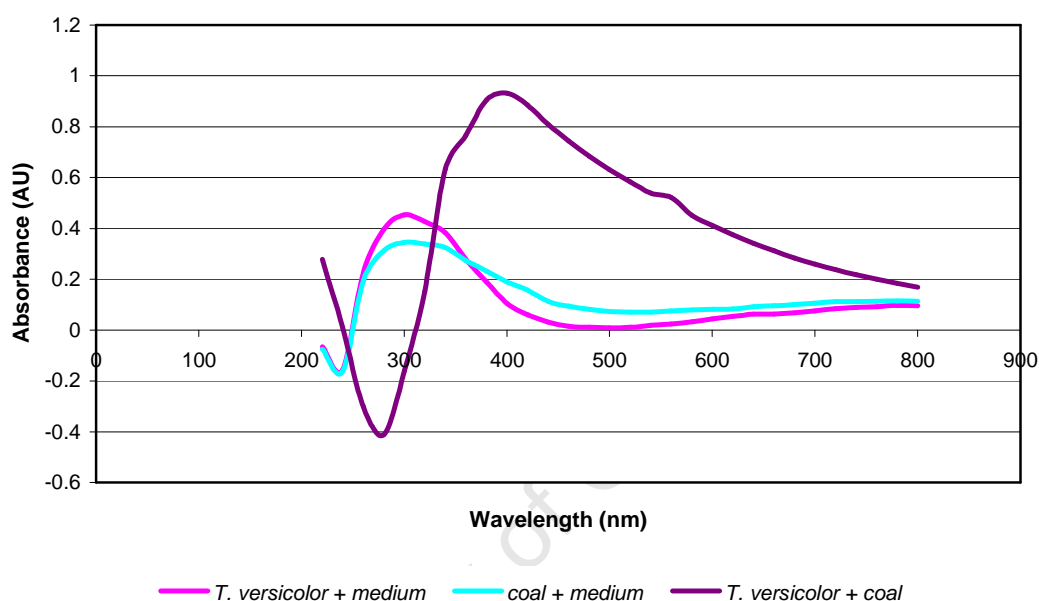


Figure 4.4 Absorption spectra of filtrate from *T. versicolor* cultures grown in the presence and absence of sub-bituminous coal (with 0.25% glucose in TDM)

HPLC profiles of the filtrates obtained from the cultures grown (with shaking) in the presence and absence of sub-bituminous coal are shown in Figure 4.5 below. Although there were some similar chromatographic peaks observed in *T. versicolor* cultures grown in the presence and absence of coal, there was also evidence of some newly formed compounds in *T. versicolor* cultures grown with coal, represented by peaks 1 and 4. These could correspond to the production of lower molecular weight fulvic acids, which were detected spectrophotometrically at 360 nm as discussed above. Analysis of methyl ethyl ketone extracts of the filtrate, from the culture grown with coal, showed that the solvent extraction was not successful, probably due to the highly polar nature of the compounds remaining in the aqueous phase.

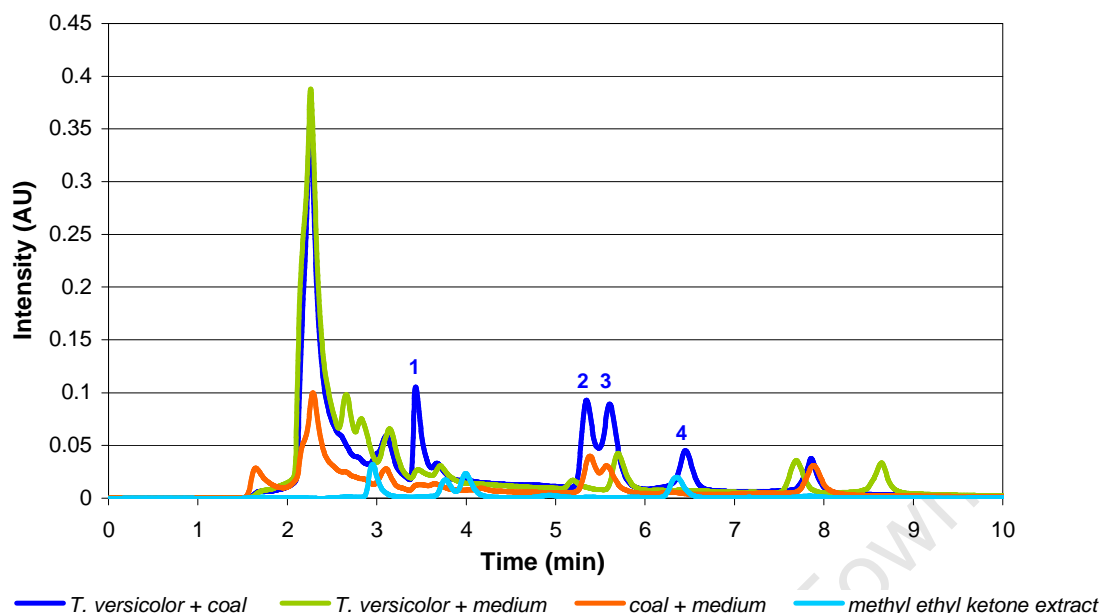


Figure 4.5 Analysis of culture filtrate, harvested from the shake flask cultures of *T. versicolor* with sub-bituminous coal, by HPLC. The mobile phase consisted of water, acetic acid and methanol (77.5:2.5:20). Peaks 1 and 4 represent newly formed compounds, while peaks 2 and 3 represent compounds also present in the 'coal + medium' control but with increased concentrations that may have resulted from the biosolubilisation of coal.

Observation of the shake flask cultures indicated attachment of the fungal hyphae to coal particles, to the extent of it being enclosed within the fungal pellets. The advantage of this is that the coal particles may be used as a solid support in a scaled-up bioprocess, entailing the use of a fluidised bed or packed bed bioreactor. This is discussed in Chapter 6. The disadvantage of this phenomenon is that quantitative measurement of coal bioconversion by dry weight analysis was not feasible.

4.3.2.2 Shake flask cultures of *T. versicolor* and *T. pubescens* with coal added after 5 days of growth

In the next set of experiments, in order to ensure that a high biomass yield and therefore, high enzyme activity, was achieved, the unaltered nutrient sufficient TDM (1% glucose) was used in shake flask cultures of *T. versicolor* and *T. pubescens*. It is known that cell wall components are involved in adhesion of fungi to their host or substrate and that the extent of adherence depends of the growth medium (Gow and Gadd, 1995). Fungal cells grown in medium with 10 times more glucose were reported to be 10 times more adherent. In the present study, in order to obtain a high biomass yield (and therefore, high laccase activity), while minimising adherence of fungal hyphae to coal, the coal was added to a 5 day-old culture,

when most of the glucose was depleted, and this coincided with day 0 of sampling. A gradual increase in the absorbance at 450 nm, suggesting the production of humic acids, was noted in both *T. versicolor* and *T. pubescens* shake flask cultures grown in the presence of coal (Figure 4.6). Similar absorbance values were obtained for the culture filtrates (from *T. versicolor* and *T. pubescens*) grown in the presence of coal to those grown in the absence of coal, but only until the eight day. Thereafter, a large difference was observed, particularly between the *T. pubescens* culture grown in the presence of coal and the culture grown without coal. Regardless of this, a net colour increase was still evident.

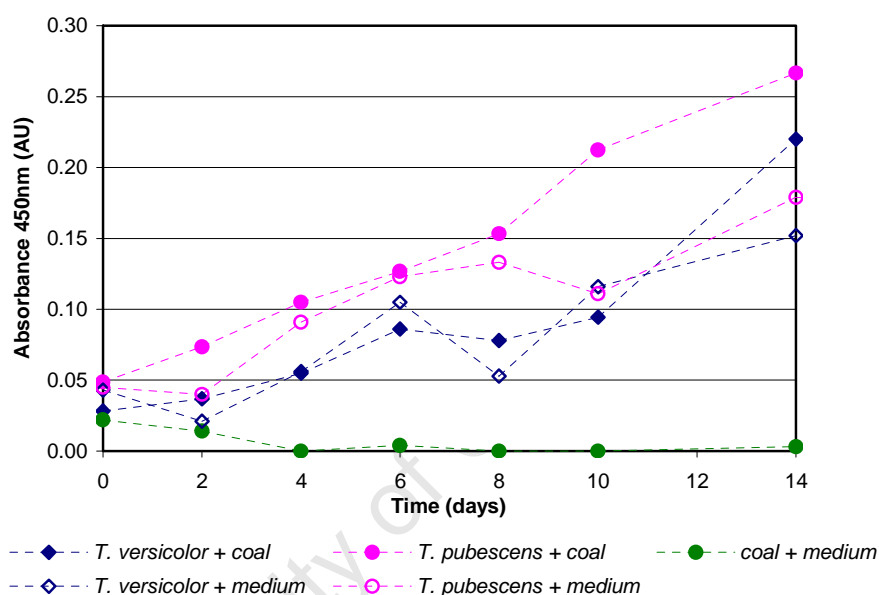


Figure 4.6 Increase in absorbance at 450 nm, indicating the release of humic acids from the bioconversion of sub-bituminous coal by *Trametes* spp, with coal added to a 5-day old culture on day 0 (Values shown are means of duplicate data. Mean standard deviations in all cases were less than 5% and therefore, error bars are not shown.)

The laccase activity found in the culture filtrate from *T. pubescens* grown in the presence and absence of coal was similar throughout the culturing time (Figure 4.7). An initial decrease in activity was measured after the addition of coal, followed by a levelling out of activity. The laccase activity displayed by *T. pubescens* was higher than that of *T. versicolor*, as was expected. No apparent trend was visible in the laccase activity of *T. versicolor* but generally, the activity was higher in cultures grown in the presence of coal. Fakoussa and Frost (1999) observed a large decrease in the measurable laccase activity when whole coal particles were added to *T. versicolor* cultures. These authors suggested that laccase was sensitive to the shear caused by the presence of coal particles, and that interference of humic acids (absorbing at 450 nm) with the laccase activity assay (measured by an increase in absorbance of the

ABTS radical at 420 nm) was likely to have occurred (Temp *et al.*, 1999; Ralph *et al.*, 1996). No LiP and very low MnP activities were detected in both *T. versicolor* and *T. pubescens* cultures under the nutrient sufficient conditions used in the present study. This was expected since LiP and MnP are usually produced under nutrient limiting conditions (Tien and Kirk, 1984; Vyas *et al.*, 1994). It should be noted that veratryl alcohol was included in the growth medium (TDM) developed for the cultivation of *Trametes* spp. (Ryan, 2003), to induce laccase activity, and that it was added in consistent concentrations to both test reaction cultures as well as control cultures.

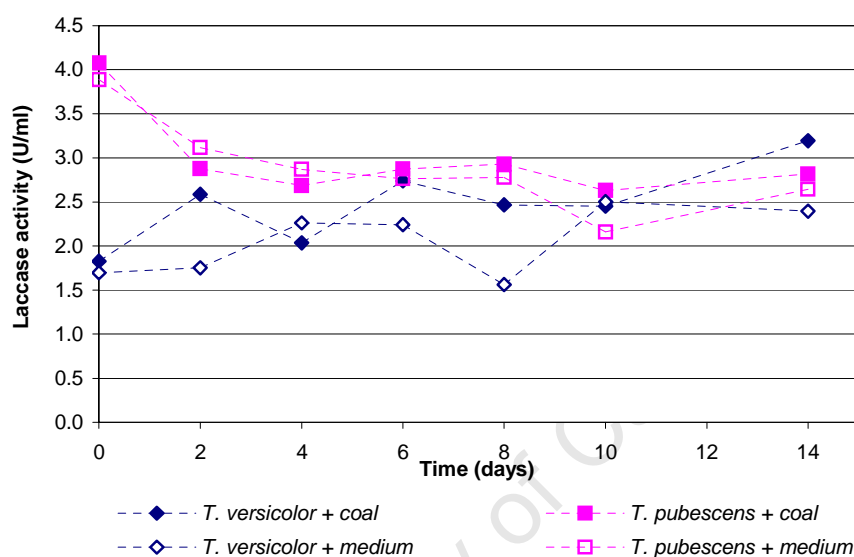


Figure 4.7 Laccase activity of shake flask cultures of *T. versicolor* and *T. pubescens* with sub-bituminous coal, added to 5-day old cultures on day 0

The pH of the culture filtrate, obtained from shake flask cultures of *T. versicolor* and *T. pubescens* with sub-bituminous coal, was measured at the end of the experiment and was found to be 7.21 ± 0.06 and 7.31 ± 0.03 for *T. versicolor* and *T. pubescens*, respectively. The pH of the control cultures *i.e.* grown in absence of coal, were approximately 7.19 suggesting that the presence of coal did not cause an increase in pH or elicit the production of alkaline materials. Torzilli and Isbister (1994) claimed that above pH 8, biosolubilisation can be attributed to increased alkalinity. Therefore, based on the results obtained in this study, the production of alkali, by *T. versicolor* and *T. pubescens*, was not induced by the presence of coal, and although the increase in pH may have contributed to coal biosolubilisation to a small extent, the production of alkali did not appear to be a mechanism through which *T. versicolor* or *T. pubescens* bioconvert sub-bituminous coal.

Coal-bound hyphae from *T. pubescens* cultures were homogenised and washed with sodium hydroxide solution in an attempt to dissolve the residual coal, so that the amount of coal biosolubilised could be quantified gravimetrically. Light microscope photographs were taken to confirm separation of coal and fungal hyphae (Figure 4.8). The images showed coal still tightly bound to hyphae. It was possible that the addition of sodium hydroxide, rather than chemically dissolving the coal, caused the release of substances from the mycelia binding the coal more tightly. This method was adapted from the method for the recovery of alkali-solubilised coal from cultures of wood-rot fungi developed by Ralph and Catchside (1997). In this procedure alkali-solubilised coal was bound to fungal cells and was precipitated by acidification. The mixture was then centrifuged and the pellet washed with alkali, re-dissolving the alkali-solubilised coal, and the cell material was separated via filtration. Though useful for alkali-solubilised coal, this method did not work for the sub-bituminous coal used in the current study due to its insolubility alkali. An alternative method allowing the quantification of amount of coal biosolubilised was therefore needed.

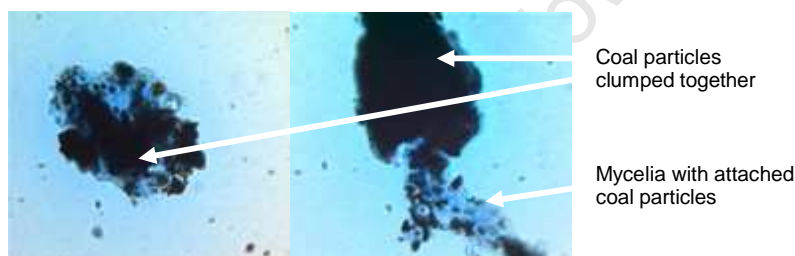


Figure 4.8 Light microscope images of coal-bound hyphae after alkali-washing and homogenisation

4.3.2.2.1 Quantification of coal bioconversion by *Trametes* spp. by acid digest-assisted elemental analysis of combined biomass and coal residue

The removal of fungal mycelia bound to coal particles, by acid digestion followed by elemental analysis of the residual coal, was used as an alternative approach to quantify the amount of solid coal converted by *T. pubescens* or *T. versicolor* in shake flask cultures. This procedure consisted of two main parts: 1) An elemental analysis of the native sub-bituminous coal (as presented in Section 2.3.1) which had not been treated with acid, and native sub-bituminous coal treated with nitric acid, that had not been subjected to biological degradation, was carried out. In this way, the amount of carbon lost due to acid digestion could be calculated. 2) An elemental analysis of the residual coal, obtained after its bioconversion by each of the *Trametes* strains, and that had been treated with acid to remove the attached fungal mycelia. The amount of coal lost due to biological degradation was then calculated by taking the amount of carbon lost due to the acid digestion itself, into account. All calculations were based on percent coal carbon (w/w), as determined by elemental analysis i.e. g carbon per 100 g of coal. The total amount of bioconverted coal

carbon was then related back to the initial amount of coal added to the shake flask cultures, and the results are represented in Table 4.3.

Approximately 22.3% loss in coal carbon was attributed to the acid digestion in control experiments using untreated coal. The amount of coal carbon converted by *T. versicolor* and *T. pubescens* was calculated to be 11.5 and 9.5 %, respectively. This equated to 67.7 and 56.1 mg of coal carbon converted per gram of coal. Reports providing quantitative data on coal bioconversion are not common, but Gockay *et al.* (2001) reported 7.59-12.93% biosolubilisation of Turkish lignite by *T. versicolor*, while Oboirien *et al.* (2008) reported a 9.3% biosolubilisation of sub-bituminous coal by *Trichoderma atroviride* ES11, determined through gravimetric analysis. The results presented in this study confirm that SA sub-bituminous coal was bioconverted by *T. versicolor* and *T. pubescens*, with conversion values comparing favourably with values reported in literature. In addition, attention must be drawn to the significance of achieving bioconversion of sub-bituminous coal, which is more recalcitrant to degradation than lignite, without having to pre-treat the coal prior to its bioconversion by *Trametes* sp. This is the first report providing quantitative data on the bioconversion of sub-bituminous coal that had not been pre-treated, by *T. versicolor* and *T. pubescens*.

Table 4.3 Elemental composition of coal residues from shake flask cultures of *Trametes* spp, after treatment with nitric acid

#	Sample	Coal residue	C	H	N	S
1	Sub-bituminous coal	Native coal (g/100g coal) ^a	59.13	3.66	1.24	0.61
	Sub-bituminous coal	After acid digestion (g/100g coal) ^b	45.96	2.48	4.58	0.05
		% lost due to digestion ^c = (a-b)/a x 100%	22.27	32.24	-269.4	91.80
2a	<i>T. versicolor</i> +coal	After acid digestion (g/100g coal) ^d	42.82	2.18	4.16	0.02
		Loss due to digestion (g/100g coal) ^{e=dxc}	9.54	0.71	1.54	0.02
		Corrected value (g/100g coal) ^{f=d+e}	52.36	2.89	5.70	0.04
		Loss due to bioconversion (g/100gcoal) ^{g=a-f}	6.77	0.77	-4.49	0.57
		% loss due to bioconversion ^{h=g/a x 100%}	11.45	21.0	-362.1	93.4
		Total loss due to bioconversion (mg/g coal)	67.7	7.7	-44.9	5.7
2b	<i>T. pubescens</i> +coal	After acid digestion (g/100g coal) ⁱ	43.77	2.27	4.35	0.02
		Loss due to digestion (g/100g coal) ^{j=i x c}	9.75	0.73	1.61	0.01
		Corrected value (g/100g coal) ^{k=i+j}	53.52	3.00	5.96	0.03
		Loss due to bioconversion (g/100g coal) ^{l=a-k}	5.61	0.66	-4.72	0.58
		% loss due to bioconversion ^{m=l/a x 100%}	9.45	18.03	-389.7	95.1
		Total loss due to bioconversion (mg/g coal)	56.1	6.6	-47.2	5.8

* All values shown are means of duplicate data. Mean standard deviations in all cases were less than 4% and therefore, errors are not shown

4.3.3 Bioconversion of lignite coal by *T. versicolor* in shake flasks

As alternative substrates, lignite coal and asphaltene are considered, based on their structures, to be more amenable and more recalcitrant to bioconversion than sub-bituminous coal, respectively (See Section 1.2.3 and Section 4.2.1). These substrates were used to gain information on the capacity of the *Trametes* sp to bioconvert different types of coal, and the work is presented in the following sections.

The bioconversion of lignite by *T. versicolor* was carried out in a 2L shake flask containing nutrient sufficient TDM (1% glucose), to enable a larger sampling volume. Lignite was added to a 7 day-old culture, when most of the glucose was depleted, which was taken as day 0 of sampling. Lignite bioconversion, measured as increase in absorbance at 460 nm and 360 nm, relating to the production of humic acids and fulvic acids, respectively, was observed over 30 days (Figure 4.9, also see Section 4.3.2.1).

In this experiment, an increase in laccase activity was observed which coincided with addition of lignite, suggesting a possible inductive effect. However, a decrease in activity was observed previously, upon addition of sub-bituminous coal to *T. versicolor* cultures in shake flasks (see Section 4.3.2.2). Thus, the increase in laccase activity observed after the addition of lignite may have resulted as a release response to components of lignite, which are more soluble than those of sub-bituminous coal. The laccase activity was stable until day 14, after which a notable decrease in activity was observed, coinciding with a high production of humic and fulvic acids, highlighting the possible inhibitory effects of humic and fulvic acids on the laccase activity. As the laccase activity decreased, the production of humic and fulvic acids levelled out. Together, these results confirmed that laccase was involved in lignite bioconversion, producing humic and fulvic acids, but these products had an inhibitory effect on its activity. In other reports, the biosolubilisation of leonardite by *T. versicolor* was attributed to laccase-like activity, based on the assertion that cyanide and azide, which are known laccase inhibitors, inhibited cell-free biosolubilisation, and that the biosolubilisation activity was partially destroyed by heating the sample, indicating the presence of enzymatic activity (Pyne *et al.*, 1987).

In the present study, the production of humic and fulvic acids from lignite was five-fold higher than that produced by *T. versicolor* growing in the presence of sub-bituminous coal, even though the apparent laccase activity was five-fold lower. Lignite coals are more oxidised, and therefore more amenable to bioconversion (Klein *et al.*, 1988). It is therefore not surprising that more humic acids were released from lignite than from sub-bituminous coal during their bioconversion by *T. versicolor*.

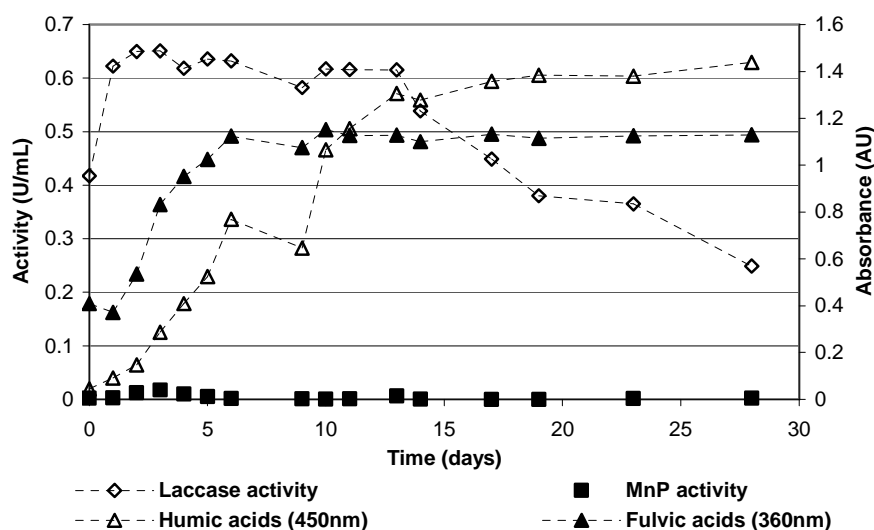


Figure 4.9 Bioconversion of lignite coal by *T. versicolor*, with coal added to a 7-day old shaken culture on day 0 (The change in laccase and MnP activity, and production of humic and fulvic acids, measured as the change in absorbance at 450 nm and 360 nm, respectively, were monitored for the duration of the experiment.)

4.3.4 Bioconversion of asphaltene by *T. pubescens* in submerged cultures

An investigation of the bioconversion of asphaltene by *T. pubescens* was undertaken because asphaltene is regarded as a suitable model compound for investigating the potential of fungi to degrade hard coal, based on its dense polyaromatic structure (See 4.2.1) (Hofrichter *et al.*, 1997). In the present study experiments with asphaltene were performed on a smaller scale due the limited amount of substrate available, initially in 1 mL vials and then in 5 mL shake flask cultures. Asphaltene ($1\text{mg}\cdot\text{mL}^{-1}$) was added to *T. pubescens* that was pre-cultivated in TDM for 5 days. The experiment was carried out for 3 weeks, and the culture filtrates were analysed by HPLC. HPLC profiles were obtained for filtrates from cultures grown in the presence and absence of asphaltene, and are shown in Figure 4.10. A small decrease in retention time was observed for a number of peaks representing compounds present in cultures grown both in the presence and absence of asphaltene, respectively. The peaks labelled 1 and 2 represented polar compounds formed or liberated from the bioconversion of asphaltene by *T. pubescens*. Tween 80, a surfactant, was added to the cultures to improve dissolution of asphaltene, but its presence did not seem to have effect on the solubilisation of asphaltene, confirmed by the HPLC profile of asphaltene and medium (containing Tween) in the absence of the fungus.

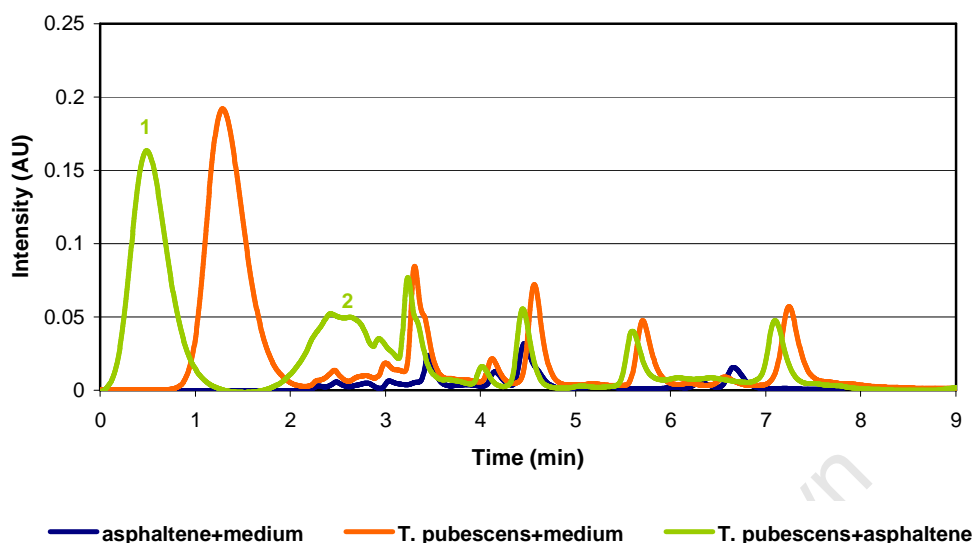


Figure 4.10 HPLC analysis of filtrate from *T. pubescens* cultures grown in the presence and absence of asphaltene

Bublitz *et al.* (1994) developed a screening system for the detection of hard coal-modifying fungi, and from this, one isolate, a *Trichoderma* species (AB2), showed the capacity to bioconvert asphaltene. An increase in carbonyl functional groups and a decrease in ether functional groups was observed by infrared spectroscopy. In another study, 2-hydroxybiphenyl, identified in a tetrahydrofuran extract by GC-MS, was the only compound liberated from the bioconversion of asphaltene by *Coprinus sclerotigenis*, a litter-decaying fungus (Hofrichter *et al.*, 1997).

In the current study, GC analysis of a tetrahydrofuran extract of the filtrate from the culture grown in the presence of asphaltene did not show the presence of any new compounds, but a notable increase in fungal biomass was observed during the incubation with asphaltene over a 3-week period. It was suggested that that bioconversion products were further metabolised to produce biomass. Fungal hyphae also adhered to asphaltene particles, as was observed in submerged cultures with lignite and sub-bituminous coal (Section 4.3.2.2). Dry weight analysis of the asphaltene-bound fungal residue showed that approximately 10% more growth was obtained in the presence of asphaltene than in its absence (Table 4.4). This value is a minimum figure, since the calculation was based on the assumption that no asphaltene was degraded and that an increase in dry weight could only be attributed to an increase in biomass. This was an important finding because it suggested that *T. pubescens* was able to degrade asphaltene and utilise it as a carbon source for growth.

Table 4.4 Gravimetric analysis of asphaltene residue before and after bioconversion by *T. pubescens* in shake flasks

Sample	Initial weight Asphaltene (mg)	Wet weight mycelia and residual asphaltene (mg)	Dry weight mycelia and residual asphaltene (mg)
Positive control^a	0	116	22
Test flask 1	5.1	417	30
Test flask 2	5.1	543	32
Negative control^b	5.3	8	6

a. No asphaltene present *b.* No fungal cells present

In a similar experiment involving the bioconversion of asphaltene by *T. pubescens*, but performed in 1 mL-vials, separation of residual asphaltene and fungal hyphae was achieved by dissolving the asphaltene in tetrahydrofuran, which was then allowed to evaporate. The measurement of fungal biomass, after the removal of asphaltene, was not accurate due to the small scale of the experiment, and the quantitative data was therefore discarded. However, scanning electron microscopy of asphaltene samples, before and after bioconversion by *T. pubescens*, showed that the asphaltene particles became more porous (Figure 4.11). Structural alterations might have been due to mechanical effects of the fungal hyphae (Hofrichter *et al.*, 1997).

The results from this study suggested that laccase-producing *T. pubescens* has the ability to grow on asphaltene, possibly causing mechanical disruption to its physical structure. *T. pubescens*, thus, has potential to degrade hard coal, since asphaltene is regarded as a basic structural unit of the hard coal molecule. Further, the potential exists for the removal or partial removal of asphaltene which is found as a high molecular weight fraction in oil. The presence of asphaltenes in crude oils is problematic due to the formation of deposits near the wellbore, and the flocculation of asphaltene, which occurs when oil solvency is reduced (Almeida, 2004). Asphaltene flocculation has detrimental effects on oil production, transportation and the refining process by plugging reservoirs, poisoning refinery catalysts and fouling production facilities. Asphaltenes are also known to be coke-precursors, causing deactivation of the catalyst during heavy oil hydrotreating. Asphaltenes are generally difficult to degrade, and conventional methods used for mitigating problems associated with asphaltene deposition are expensive, including mechanical removal and/or the use of chemicals, i.e. organic solvents, surfactants, polyelectrolytes, oxidising agents, etc (Trejo *et al.*, 2004). Thus, the removal or partial removal of asphaltene through the bioconversion by *T. pubescens* would be economically attractive to the petroleum industry. The experimental results described here have led to the proposal of a process for the biodegradation of asphaltenes (Vengadajellum and Burton, 2008 Patent ZA 2008/05093).

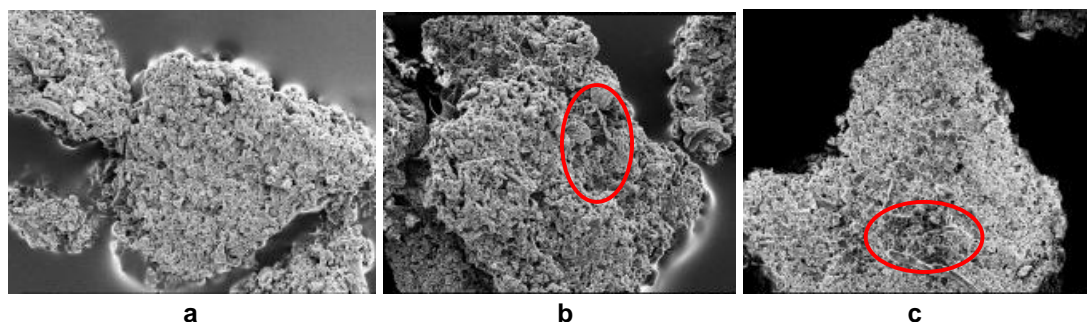


Figure 4.11 SEM analysis of (a) asphaltene residue from control reaction (asphaltene and growth medium), (b-c) asphaltene residue after treatment with *T. pubescens* (all 500x magnification) (The red circles indicate porous areas that may have resulted from mechanical effects of fungal hyphae.)

4.4 CONCLUSIONS

In work presented in this chapter, sub-bituminous coal, lignite and asphaltene were successfully bioconverted by cultures of *Trametes* spp. in shake flasks. The biosolubilisation of sub-bituminous coal was evident through the conversion of powdered coal to a liquid substance above the hyphal mat in static cultures of *T. versicolor*. HPLC analysis of biosolubilised coal extracts, compared with those of untreated coal, indicated structural changes to chromophores, conferring altered UV absorption properties, and suggested the production of more polar compounds.

In shake flask culture experiments with *T. versicolor* and *T. pubescens*, coal bioconversion was evidenced by the production of humic and fulvic acids (measured spectrophotometrically); the formation of aromatic compounds (measured by HPLC) and loss in coal carbon (measured by elemental composition).

Of the oxidase enzymes, including laccase, MnP and LiP, laccase was predominantly produced under the nutrient sufficient conditions provided during the bioconversion of coal by *Trametes* spp. A correlation existed between the glucose concentration in the growth medium and the laccase activity of *T. versicolor* when grown in the presence of sub-bituminous coal in shake flasks. Higher glucose concentrations (and therefore higher biomass yields) led to increased laccase activity.

Humic and fulvic acids were produced from the bioconversion of sub-bituminous coal and lignite by *Trametes* spp. in shake flasks. However, the production of humic acids was inversely correlated with laccase activity in *T. versicolor* and *T. pubescens* cultures, suggesting that a higher laccase activity (and biomass yield) increased mineralisation and result in lower yields of humic acids. Similarly, with lignite

bioconversions by *T. versicolor*, laccase activity decreased rapidly, coinciding with the production of high levels of humic acid. Thus, the direct inhibition of the enzyme or the protein binding capacity of coal particles and/or humic acids may have resulted in lower measurable laccase activity.

Gravimetric analysis of residual coal, allowing the quantitative determination of coal bioconversion by *Trametes* spp., was not possible due to the attachment of fungal hyphae to coal particles during the bioconversion of sub-bituminous coal in shake flask cultures. Thus, the acid digest-assisted elemental analysis method was developed, which included the removal of fungal mycelia from coal through digestion with concentrated nitric acid. Subsequently, the bioconversion of sub-bituminous coal by loss of carbon was measured by elemental analysis. 11.5 and 9.5 % coal carbon was degraded by *T. versicolor* and *T. pubescens*, respectively. These values compared favourably with those cited in literature (Gockay *et al.*, 2001; Oboirien *et al.*, 2008), and was the first report providing quantitative data on the bioconversion of sub-bituminous coal, that had not been pre-treated, by *T. versicolor* and *T. pubescens*.

T. pubescens was active towards asphaltene, as evidenced by the increase in porosity in treated asphaltene particles, as well as an increase in colour of the culture filtrate. However, with the exception of some polar compounds separated by HPLC, no noteworthy new products were observed. This, together with the notable increase in biomass observed when cells were grown in the presence of asphaltene, compared with those grown in its absence, could signify the mineralisation of products to form more biomass. Mechanical disruption of asphaltene particles could not be discounted as a cause of degradation by *T. pubescens*, due to the close physical interaction between fungal hyphae and asphaltene particles. *T. pubescens*, thus, has potential to degrade hard coal since asphaltene is regarded as a basic structural unit of the hard coal molecule, and the potential also exists for the removal or partial removal of asphaltene, from oil, which would be economically attractive to the petroleum industry.

The ability of *Trametes* spp. to bioconvert low rank coals, such as lignite and sub-bituminous coal and the more recalcitrant asphaltene, without requiring expensive chemical oxidative pre-treatment is a key finding because it demonstrated the versatility of these strains in degrading a range of differently ranked coals. Further, bioconversion of coal by *Trametes* spp. would be more economically viable since chemical oxidative pre-treatment of the coal is not required, thus reducing the cost of the bioprocess. Future work to be considered should involve the investigation of the role of laccase and its mediators in the degradation of coal. An effective bioreactor configuration for the bioconversion of sub-bituminous coal by *Trametes* sp would enable a more stringent determination of the degradative capacity of these strains, in terms of kinetic rate constants. These areas were the foci of the research which is presented in Chapters 5 and 6, respectively.

CHAPTER 5: BIOCONVERSION OF SUB-BITUMINOUS COAL BY AN EXTRACELLULAR, CELL-FREE EXTRACT FROM *TRAMETES PUBESCENS*

5.1 INTRODUCTION

The bioconversion of lignite and sub-bituminous coal by *Trametes versicolor* and *Trametes pubescens* in liquid culture has been demonstrated and was discussed in the previous chapter. It was concluded that laccase was the oxidative enzyme predominantly produced under the nutrient-sufficient conditions provided. We therefore proposed that laccase was responsible for the bioconversion of coal to produce humic and fulvic acids. However, the degradation of coal might also be partly due to physical interaction between fungal hyphae and solid substrate particles and this close association might have facilitated mineralisation of the intermediate products. The objective of the work reported in this section was therefore to test the effect of a crude, cell-free preparation from *T. pubescens* on the bioconversion of sub-bituminous coal. In this way, the role of crude laccase in the bioconversion of coal could be confirmed and the effect of cell-free system on the recovery of humic acids produced could be measured. Further, the importance of the physical interaction between fungal hyphae and the coal particles on the bioconversion of sub-bituminous coal by *T. pubescens* could be assessed.

Microorganisms bioconvert coal through different mechanisms which have been discussed in detail in Chapter 1 (see Section 1.3.3). Several studies have been carried out to elucidate the mechanisms by which *T. versicolor* bioconverts lignite coal but conflicting evidence has been presented in literature. The production of chelators and the action of oxidase enzymes in the bioconversion of leonardite (highly weathered lignite) by *T. versicolor* were mechanisms commonly reported on. Although non-enzymatic solubilisation by cell-free filtrates from *T. versicolor* was affected by the pH of the buffer solution (Cohen *et al.*, 1987), the production of alkaline materials as a mechanism of coal biosolubilisation has not been studied in detail in this strain, or in other white-rot fungi.

In general, the biosolubilisation of coal is not always accompanied by its depolymerisation, but certain fungi have shown the ability to biosolubilise and depolymerise lignite albeit under different conditions and by different mechanisms (Hofrichter *et al.*, 1997; Torzilli and Isbister, 1994; Cohen and Gabriel, 1982; Fakoussa and Frost, 1996). The action of laccase in the biosolubilisation of lignite was implicated in early research by Cohen *et al.* (1987) and Pyne *et al.* (1987), but its role in the biosolubilisation process has been disputed, because conditions under which biosolubilisation occurred (e.g. pH 7-10) were generally unfavourable for the production of oxidases. However, laccase has been shown to be actively involved in the decolourisation and depolymerisation of coal-derived humic acids (Fakoussa and Frost, 1996).

Although laccase catalyses the oxidation of compounds by one electron abstraction, giving rise to radicals which can subsequently polymerise, it is also capable of carbon-carbon and carbon-oxygen cleavage (Majcherecyk *et al.*, 1999). In some cases, like the bleaching of hardwood kraft pulp, the presence of certain mediators was necessary to achieve biosolubilisation and depolymerisation of the lignin (Bourbonnais and Paice, 1990). A mediator is usually a small molecule that acts as an electron shuttle. Once it is oxidised by the enzyme, it can act as a diffusible oxidant of any substrate that could not enter the enzyme pocket due to its size (Claus, 2004; Rittstiet *et al.*, 2003). Veratryl alcohol (3, 4-dimethoxybenzyl alcohol) is a secondary metabolite that is synthesised *de novo*, from L-phenylalanine, by several white-rot fungi (Zapanta and Tien, 1997), and is a known low molecular weight redox mediator involved in the catalysis of substrates by lignin peroxidase, allowing lignin peroxidase to oxidise compounds of high redox potential (see Section 1.3.4.). The presence of veratryl alcohol has also been shown to play a role in enhancing the production of laccase in certain fungi, and by acting as a redox mediator, has expanded the catalytic activity of laccase towards substrates that are more difficult to oxidise (Dekker and Barbosa, 2001; Canterella *et al.*, 2003). Laccase-mediator systems, such as this, have thus increased the value of using laccase in comparison to the more powerful peroxidases, which have higher redox potentials.

However, the redox potential of laccase varies depending on the source of the enzyme (Osma *et al.*, 2006). Recently, *Trametes pubescens* has been identified as an excellent source of laccase, which has been classified as a high redox potential laccase (Galhaup and Haltrich, 2001; Schleev *et al.*, 2007). Thus, the decolourisation (and thus, depolymerisation) of the anthraquinonic dye, Remazol Brilliant Blue R (RBBR) by extracellular fluid from *T. pubescens*, in the absence of a redox mediator, has been reported (Osma *et al.*, 2006). Bearing this in mind in relation to the depolymerisation of coal macromolecules, and in meeting with the long term goal of the current research project, which was to produce low molecular mass compounds from the bioconversion of low rank coal, the ability of laccase from *T. pubescens* to depolymerise sub-bituminous coal was investigated.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Reagents were all of analytical or HPLC grade as required. Sub-bituminous coal (SASOL, 2001) was not pre-treated other than sterilisation by autoclaving at 120°C.

5.2.2 Preparation of extracellular, cell-free extract from *T. pubescens*

T. pubescens was grown in a draught-tube internal loop airlift reactor (ALR) (see Section 6.2.6 for ALR specifications). The starter culture was prepared in 2-L Erlenmeyer flasks containing approximately 315 mL *Trametes* defined medium (TDM) (Appendix A) and a 35 mL inoculum (10%). The inoculum was prepared by homogenising the solid media culture of *T. pubescens* in 200 mL TDM.

The ALR vessel was made from perspex, and it could therefore not be autoclaved. Sterilisation was performed by filling the vessel and all tubing with 10% Sodium hyperchlorite solution (bleach) and allowing circulation in the vessel for 24 hours. Sterile air was supplied allowing a continuous positive air pressure. The vessel was subsequently rinsed with sterile, distilled water for 24 hours before autoclaved TDM was pumped in. The reactor was filled with approximately 3.0 L TDM containing 2 mL antifoam 204 (Sigma). 200 mL of the starter culture was added (7% inoculum). Veratryl alcohol (24.4 μ M), included in the TDM, was used to induce laccase activity produced by *T. pubescens*. The working volume of the reactor with gas hold-up was 3.5 L.

After 5 days of growth, the air was turned off and the biomass was allowed to settle. The supernatant was siphoned out the top of the reactor under aseptic conditions. The supernatant was not subjected to sterile filtration due to the large volume and the need to minimise contamination, which would have been introduced by transferring the solution to additional vessels. After measuring the laccase activity, the supernatant was not treated further and was used as the cell-free extract in bioconversion experiment as described below.

5.2.3 Bioconversion of sub-bituminous coal by an extracellular, cell-free extract from *T. pubescens*

250 mL of the cell-free extract (culture supernatant) was added aseptically to 1L-Erlenmeyer flasks, each containing 1.25 g of sterile, sub-bituminous coal. The coal was fractionated by dry-sieving to obtain particles of 212–300 μ m in size. The control contained coal and supernatant that had been autoclaved (heat sterilised) to denature any enzymes. The flasks were incubated at 160 rpm at 28 °C for 25 days.

1.5 mL samples were taken on day 0, 3, 6, 9 and 25 and were analysed for laccase activity, humic acids (A_{450} nm) and total phenolic content. Reverse-phase HPLC at 280 nm was used to monitor the production of aromatic compounds and the relative molecular mass distribution of the product was determined by HPLC–size exclusion chromatography (SEC). On day 25, the residual coal was obtained by filtration, dried overnight at 80°C and its elemental composition was determined. The supernatant was acidified to pH 1.5 using 6N HCl to precipitate humic acids. The humic acid precipitate was obtained by filtration under vacuum, dried overnight at 80°C and weighed. It was then re-dissolved in 0.1M NaOH solution. The absorbance of these samples was measured at 450 nm and UV-Visible spectrophotometric scans were compared to the profile of a humic acid standard.

5.2.4 Laccase assay

Laccase activity was determined by following the oxidation of 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Wolfenden and Willson, 1982). 0.17 mL of filtrate was added to 2.5 mL sodium acetate buffer (0.1 M, pH 5) and 0.33 mL of the ABTS solution (5 mM) in a 3 mL cuvette (Roy-Arcand and Archibald, 1991). The change in absorbance was read at 420 nm over time using a Unicam Helios Alpha/Beta UV-Vis spectrophotometer. An extinction coefficient of $36\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ (Roy-Arcand and Archibald, 1991) was used to determine laccase activity, where 1 U of activity is equivalent to the amount of laccase required to catalyse the conversion of 1 μmol of substrate per minute.

5.2.5 High performance liquid chromatography (HPLC)

Reverse-phase HPLC was employed using a LaChrom System (Merck, Germany) with UV detection (L-7400) using reverse-phase columns: (1) 5 μm C18 Phenomenex Envirosep PP (125mm x 4.6mm) and (2) 5 μm C18 Waters Spherisorb (250mm x 4.6mm) and peaks were detected and analysed using a D-7000 HPLC System Manager. The mobile phase consisted of a solution of water, acetic acid and methanol (77.5:2.5: 20). The concentration of veratryl alcohol was determined by analysing standards of known concentration and constructing a calibration curve of concentration vs. peak area (Appendix B, Figure B2).

5.2.6 HPLC- Size exclusion chromatography (SEC)

The molecular weight distribution of samples from the bioconversion of coal by neat, cell-free filtrates from *T. pubescens* was determined by size exclusion chromatography. A Shodex HS-7M (300mm x 7.8mm) column was attached to a LaChrom System (as above) with UV and RI detection (Agilent 1100 series). Peaks were detected and analysed using a D-7000 HPLC System Manager. Filtered and degassed distilled water was the mobile phase. The column was calibrated with dextran standards ranging in size from 2000 kDa to 24 kDa. (The calibration curve is shown in Appendix B, Figure B1.)

5.2.7 Spectrophotometry

Samples were placed in quartz cuvettes and UV spectrum scans were taken over a wavelength range of 200 nm to 600 nm using a Unicam Helios Alpha/Beta UV-Vis spectrophotometer (organic and aqueous fractions). Sodium acetate buffer solution was used as a blank.

5.2.8 Microanalysis

Microanalysis of coal was conducted by the Department of Chemistry, University of Cape Town. The method employs a Thermoflash 1112 series C, H, N, S and O analyser with combustion analysis in pure oxygen and GC column separation using helium gas. Compounds were detected using differential thermal conductivity.

5.3 RESULTS AND DISCUSSION

The bioconversion of sub-bituminous coal by a cell-free filtrate containing high laccase activity, from *T. pubescens*, pre-cultivated in a draft-tube airlift reactor, was investigated. The 5-day old culture filtrate, which had a laccase activity of approximately 4 U m L^{-1} at the start of the experiment, was added to sterile coal, and was incubated with shaking for about 2 weeks. The results showed that sub-bituminous coal was bioconverted by the cell-free filtrate, and that humic acids and other low molecular mass compounds were produced through depolymerisation reactions, which coincided with high laccase activity. These results are discussed below.

5.3.1 Production of humic acids and effects on laccase activity during the bioconversion of sub-bituminous coal by a cell-free preparation from *T. pubescens*

During the treatment of sub-bituminous coal with the cell-free preparation from *T. pubescens*, an increase in the absorbance of the supernatant samples at 450 nm, over time, indicated the production of humic acid (Figure 5.1). A much smaller (but not negligible) increase in the absorbance (450 nm) was also observed in the control sample, containing autoclaved culture filtrate and sterile coal, which suggested that laccase from *T. pubescens* was not solely responsible for the production of humic substances. Rather, the effect of other mechanisms of coal bioconversion, such as the presence of chelators and/or biosurfactants, could not be discounted, particularly since *T. versicolor* was reportedly capable of both enzymatic and non-enzymatic bioconversion of lignite (Cohen *et al.*, 1990, Fredrickson *et al.*, 1990, Ralph and Catchside, 1997). However, non-enzymatic mechanisms involved in the bioconversion of lignite coal, such as chelation and the production of alkaline substances, were more prominent in highly oxidised coals such as leonardite or coals that have been subjected to oxidative pre-treatment (reviewed by Hofrichter and Fakoussa, 2001; Quigley *et al.*, 1989; Fakoussa, 1994).

The pH of the cell-free filtrate prior to the addition of coal was 6.5. Analysis the sample supernatant pH, at the end of the reaction, showed that it had increased to 7.27 in the test sample, but had decreased to 5.72 in the control sample (see Figure 5.2), and suggested that enzymatic and non-enzymatic reactions had contributed to a pH increase and decrease, respectively.

The laccase activity was observed to decrease over the same time period as the increasing production of humic acids. This result was not surprising since humic acids are known to inhibit the enzyme (Claus and Filip, 1990), and the adsorption of laccase to humic substances may have also contributed to a lower measurable laccase activity (Baldrain, 2006). However, the stability of laccase over the 2 week reaction period was not tested; thus the loss in activity may have been due to denaturation over time. The alkaline pH, which is not optimal for laccases, may have been inhibitory, and/or could have had an effect on the rate of catalysis of the substrate (ABTS) used in the assay resulting in the decreased activity observed. In

addition to measuring the laccase activity in the control flask, containing autoclaved culture filtrate and sterile coal, the laccase activity in a second control flask, containing the culture filtrate alone, should have been monitored and is therefore recommended for future experiments, involving the validation of the negative effects of the presence of humic acids and pH on laccase activity during the bioconversion of coal.

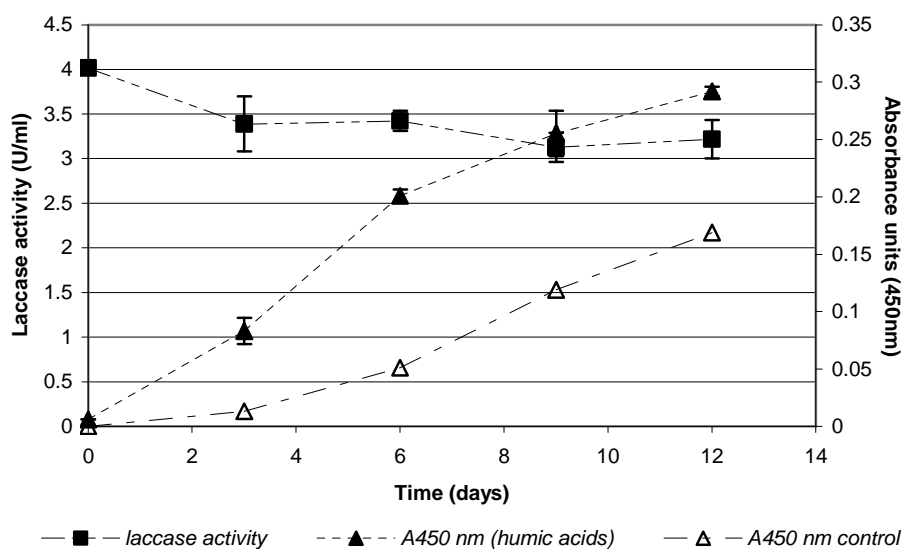


Figure 5.1 Production of humic acids, and laccase activity during the bioconversion of sub-bituminous coal by a cell-free preparation from *T. pubescens*

5.3.2 Acid precipitation of humic acids produced during the bioconversion of sub-bituminous coal by a cell-free preparation from *T. pubescens*

In order to obtain a quantitative estimate of the humic acid produced during the bioconversion of sub-bituminous coal by the cell-free extract from *T. pubescens*, the reaction supernatant was harvested at the end of the reaction, and was treated with concentrated hydrochloric acid to precipitate the humic acids. Thus, 0.052g of precipitated product was obtained from the reaction mixture, and 0.032g was obtained from the control, each originally containing 1.25 g of coal (Figure 5.2).

The mass of the precipitate was likely to have included proteins and other metabolites that were also precipitated by acid. For this reason, the precipitate was dissolved in a 0.1M sodium hydroxide solution and its UV-Visible spectral scan was compared to that of a humic acid standard (0.5 mg mL⁻¹) solution.

The similarities of the scans are shown clearly in Figure 5.3, confirming the presence of humic acids in the precipitate.

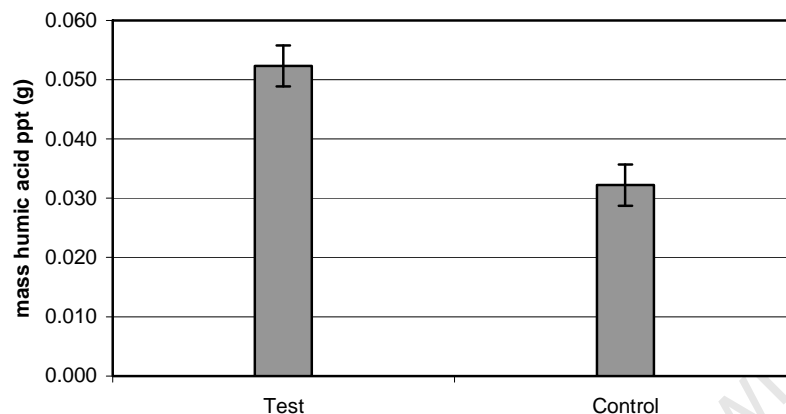


Figure 5.2 Mass of humic acid precipitate (columns) obtained after the treatment of sub-bituminous coal with a cell-free culture filtrate from *T. pubescens*

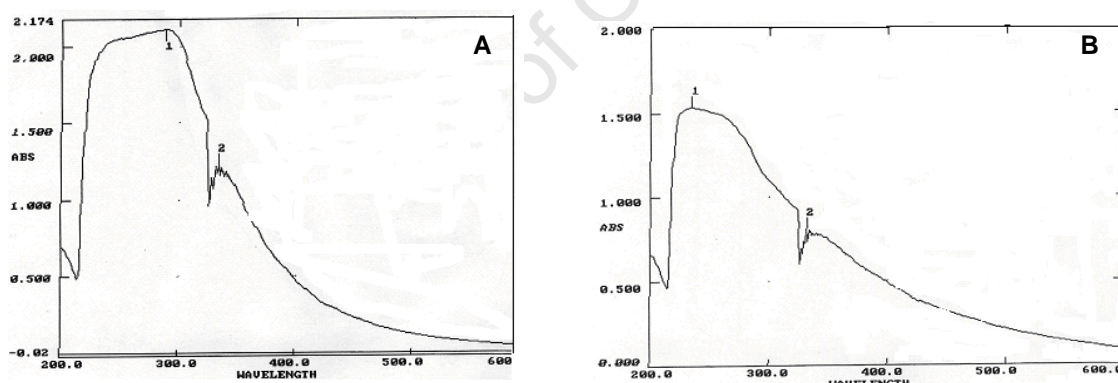


Figure 5.3 Spectral scan of humic acid precipitate, obtained from the bioconversion of sub-bituminous coal by the cell-free filtrate from *T. pubescens* (10 x dilution)(A) compared to a humic acid standard (100x dilution) (B), each dissolved in sodium hydroxide

In addition to comparing the spectral scans, the absorbance (at 450 nm) of each solution was used to calculate the actual amount of humic acids present in the precipitate. These results, presented in Table 5.1 below, showed that only 7.3% of the weight of the precipitated product, obtained from the bioconversion of sub-bituminous coal, was accounted for as humic acids. Thus, approximately 8 mg of humic acids were produced per gram of coal bioconverted by the cell-free filtrate from *T. pubescens*,

and ten times more humic acids were produced when active enzymes (laccase) were present than in the control (0.8 mg/g coal), in which enzymes were denatured by autoclaving, indicating that laccase was involved in the bioconversion of sub-bituminous coal to produce humic acids.

Table 5.1 Relative amounts of humic acids produced from the bioconversion of sub-bituminous coal by a cell-free filtrate from *T. pubescens*, calculated from the absorbance obtained from a pure humic acid standard solution

Sample	Absorbance 450 nm (AU)	Mass of humic acid (mg) in 10 mL	Mass of humic acid (mg) in 250 mL	Mass of humic acid/ mass of coal (mg/g)*
Humic acid std (100x dilution)	0.33	5.2	N/A	N/A
Reaction mixture	2.42	0.38	9.5	7.6
Control	0.24	0.038	0.95	0.76

* Initial amount of coal added = 1.25g

5.3.3 Bioconversion of coal carbon by a cell-free preparation from *T. pubescens*, measured by elemental analysis of the residual coal

The amount of coal carbon bioconverted during the bioconversion of sub-bituminous coal, by the cell-free filtrate from *T. pubescens*, was quantified by comparing the elemental composition of the residual coal obtained after the reaction with that of the control (containing autoclaved culture filtrate and sterile coal), and that of untreated coal (Figure 5.4). A reduction from 59.13% coal carbon to 53.37% was obtained, accounting for an overall loss of 9.7% coal carbon *i.e.* 9.7 g per 100 g of coal carbon was lost due to bioconversion by the cell-free filtrate from *T. pubescens*. Only 0.83% loss in coal carbon was observed in the control. This is a noteworthy result because 97 mg per gram of coal was degraded and only 8 mg of that was present as humic acids, suggesting that depolymerisation of humic acids occurred, resulting in lower molecular mass compounds that were subsequently mineralised. In addition to this, analysis of the culture filtrate by HPLC did not show the formation of new aromatic compounds, also suggesting that mineralisation reactions had occurred. It is thus proposed that sub-bituminous coal was depolymerised by the laccase present in the cell-free filtrate obtained from *T. pubescens*, initially producing humic acids, which were further depolymerised to form compounds of lower molecular mass that were later mineralised.

A small increase in the hydrogen content and a decrease in the nitrogen content of the residual coal, as compared with the untreated coal, was also observed. However, a large increase in the oxygen content was observed, which is consistent with an oxidative attack on the coal (Zavgordnyaya *et al.*, 2002).

Since the supernatant used as the cell-free preparation was not filter-sterilised, it is possible that small amounts of fungal mycelia were present, which may have been responsible for mineralising the lower molecular mass compounds produced. To ensure that a true cell-free preparation was used, the supernatant should have been sterilised by filtration to prevent renewed growth of the fungus. Thus, future work should include a repeat of these experiments including the above precaution measures.

During the bioconversion of sub-bituminous coal by *T. pubescens* in shake flasks, 9.5% coal bioconversion was achieved (Section 4.3.2.2.1), compared with the 9.7% coal bioconversion obtained from the treatment of sub-bituminous coal by the cell-free filtrate from *T. pubescens*. Thus, the physical interaction between coal particles and fungal hyphae did not appear to have a critical effect on the total amount of coal bioconverted. However, when comparing the humic acid production (by measuring the increase in absorbance at 450 nm), twice as much humic acid was produced when sub-bituminous coal was treated with the cell-free filtrate from *T. pubescens* (in the absence of fungal hyphae) (see Figure 4.6, Section 4.3.2.2), than in the shake flask cultures of *T. pubescens*, which exhibited a lower laccase activity than the cell-free filtrate. Thus, the mineralisation of humic acids during the bioconversion of sub-bituminous coal occurred to larger extent in the presence of fungal hyphae, confirming that the physical interaction between coal particles and fungal hyphae had an effect on the fate of coal carbon.

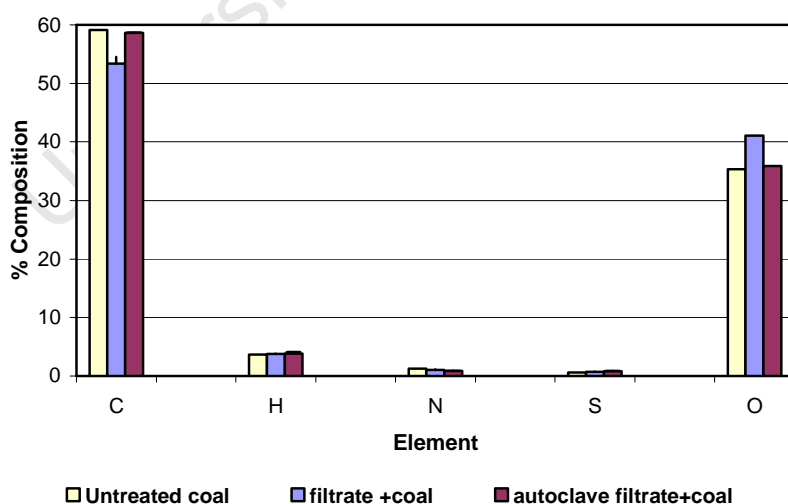


Figure 5.4 Elemental composition of sub-bituminous coal before and after treatment with a cell-free filtrate from *T. pubescens* (% Oxygen obtained by difference)

5.3.4 Depolymerisation of coal by a cell-free preparation from *T. pubescens*, monitored by HPLC-size exclusion chromatography (HPLC-SEC)

The acid precipitated product obtained from the bioconversion of sub-bituminous coal by the cell-free filtrate from *T. pubescens* was analysed by HPLC-SEC. Here, the relative molecular mass of products was estimated from the velocity at which compounds passed through a porous gel relative to dextran standards of different known molecular masses. In size exclusion chromatography the larger compounds elute first. The molecular mass distribution of the precipitate was compared with that of the control (autoclaved filtrate and coal), and with the humic acid standard (of 100-fold dilution). The SEC analyses indicated that sub-bituminous coal was depolymerised by the cell-free filtrate from *T. pubescens* to form humic substances and other polymers of smaller molecular mass (Figure 5.5). The peak representing humic acid was clearly identified (peak 1, 2 and 3), since it eluted first and represented the largest polymers, of approximately 1 mDa to 840 kDa. Although the same peak was visible in the control sample, in comparison with the test sample, this peak was unimportant in terms of size. (It should be noted that the test sample was diluted 10-fold).

This result was consistent with the spectrophotometric results discussed earlier (Section 5.3.2) and with results reported in literature; the estimated molecular mass of coal macromolecules obtained from the bioconversion of lignite by fungal strains, isolated from a lignite open cast mine, ranged from 240 to 1000 kDa (Willmann & Fakoussa, 1997). Coal degradation in this case was attributed to the action of peroxidase and/or laccase. However, these authors were unable to demonstrate the degradation of coal with purified peroxidase and enriched laccase *in vitro*.

In the current study, another notable peak observed in the control sample had a molecular weight of approximately 280 kDa (peak 6), representing a product of a non-enzymatic mechanism. Non-enzymatic solubilisation of coal may result in the production of humates through polymerisation of compounds leached from coal rather than the depolymerisation of coal macromolecules (Hofrichter and Fakoussa, 2001). Interestingly, it was not observed in the sample containing laccase, suggesting that humates resulting from non-enzymatic solubilisation of coal were also depolymerised by laccase during the bioconversion of sub-bituminous coal by the cell-free filtrate from *T. pubescens*.

The molecular mass distribution of the precipitate obtained from the culture filtrate containing coal showed the highest number of peaks, representing products of smaller molecular mass (peaks 4, 5, 7, 8 and 9). A summary of the estimated molecular masses is recorded in Table 5.2. We therefore concluded that humic acids were produced during the bioconversion of sub-bituminous coal by cell-free filtrates from *T. pubescens* containing high laccase activity, and that subsequent depolymerisation of humic acids by laccase produced products of lower molecular mass. This was a key finding because the depolymerisation of native sub-bituminous coal by laccase (and *T. pubescens*) has not been

reported before. In literature reporting the bioconversion of coal via depolymerisation reactions, only the depolymerisation of coal-derived humic acids, which had been extracted chemically with alkali solutions, has been described.

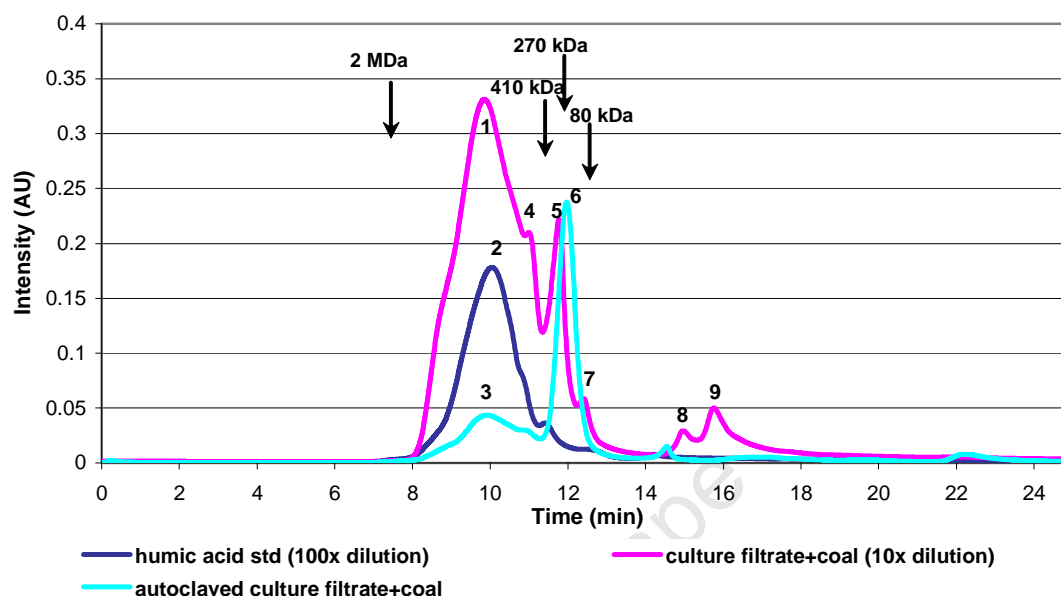


Figure 5.5 Molecular mass distribution of the acid precipitated product from the bioconversion of sub-bituminous coal by a cell-free filtrate from *T. pubescens*

Table 5.2 Relative molecular mass of compounds produced during the bioconversion of sub-bituminous coal by the cell-free filtrates from *T. pubescens* (Dextran polymers of varying molecular masses were used to construct a standard curve (Appendix B).)

Peak no.*	Retention time (min)	Molecular mass (Da)
1	9.43	901 900
2	10.11	847 667
3	10.13	841 000
4	11.04	536 333
5	11.99	219 667
6	11.81	281 000
7	12.47	60 667
8	15.07	<60 000
9	15.83	<60 000

* Figure 5.5

In the experiments performed throughout this project, veratryl alcohol had been added to the growth medium (TDM) used to cultivate *Trametes* spp., to induce laccase activity, as described in Section 4.3.2.2. Similarly, veratryl alcohol had been included in the growth medium during the preparation of the cell-free extract from *T. pubescens*, to induce laccase activity, but it might also have acted as a mediator for laccase or may have been co-oxidised in the depolymerisation of the sub-bituminous coal. The presence of veratryl alcohol in the cell-free filtrate harvested from *T. pubescens* was confirmed by HPLC (Figure 5.6). The initial concentration of veratryl alcohol added in the growth medium used to cultivate *T. pubescens*, was 24.4 μM , but the concentration of veratryl alcohol in the culture filtrate, harvested on day 5 of the cultivation, to be used in the bioconversion of coal, had increased to 74.4 μM , suggesting that the fungus had also synthesised veratryl alcohol *de novo*. Veratryl alcohol was probably synthesised from the L-phenylalanine (as discussed in Section 5.1), present in the peptone which was added as a nitrogen source to the TDM.

The presence of veratryl alcohol in the filtrate obtained from the test solution was almost undetectable by HPLC analysis, but the concentration of veratryl alcohol in the control (containing autoclaved filtrate and coal) was almost unchanged (Figure 5.6), suggesting that it had been used up in the depolymerisation of sub-bituminous coal by the cell-free filtrate from *T. pubescens*. Veratryl alcohol may have acted as the diffusible oxidant, causing direct carbon-carbon cleavage of the coal macromolecules, which could not be accessed by laccase itself, due to the large size of the macromolecules. This mechanism of depolymerisation has previously been described for the depolymerisation of lignin by laccase (Bourbonnais and Paice, 1990).

The possible involvement of veratryl alcohol in the depolymerisation of sub-bituminous coal was only realised once samples had already been analysed by HPLC and were discarded. Ideally, an internal standard should have been added in order to accurately quantify the amount of veratryl alcohol that was consumed or produced. Further experiments are necessary in order to confirm the role of veratryl alcohol in the depolymerisation of coal by *T. pubescens*. For an example, confirmation of the incorporation of veratryl alcohol in the depolymerisation reaction may be carried out by using C^{14} –radiolabelled veratryl alcohol in coal bioconversion experiments with *T. pubescens*.

Altogether, these results suggested that, during the bioconversion of sub-bituminous coal by the cell-free filtrate from *T. pubescens*, the depolymerisation reaction was facilitated by laccase, and was possibly assisted by veratryl alcohol acting as a mediator.

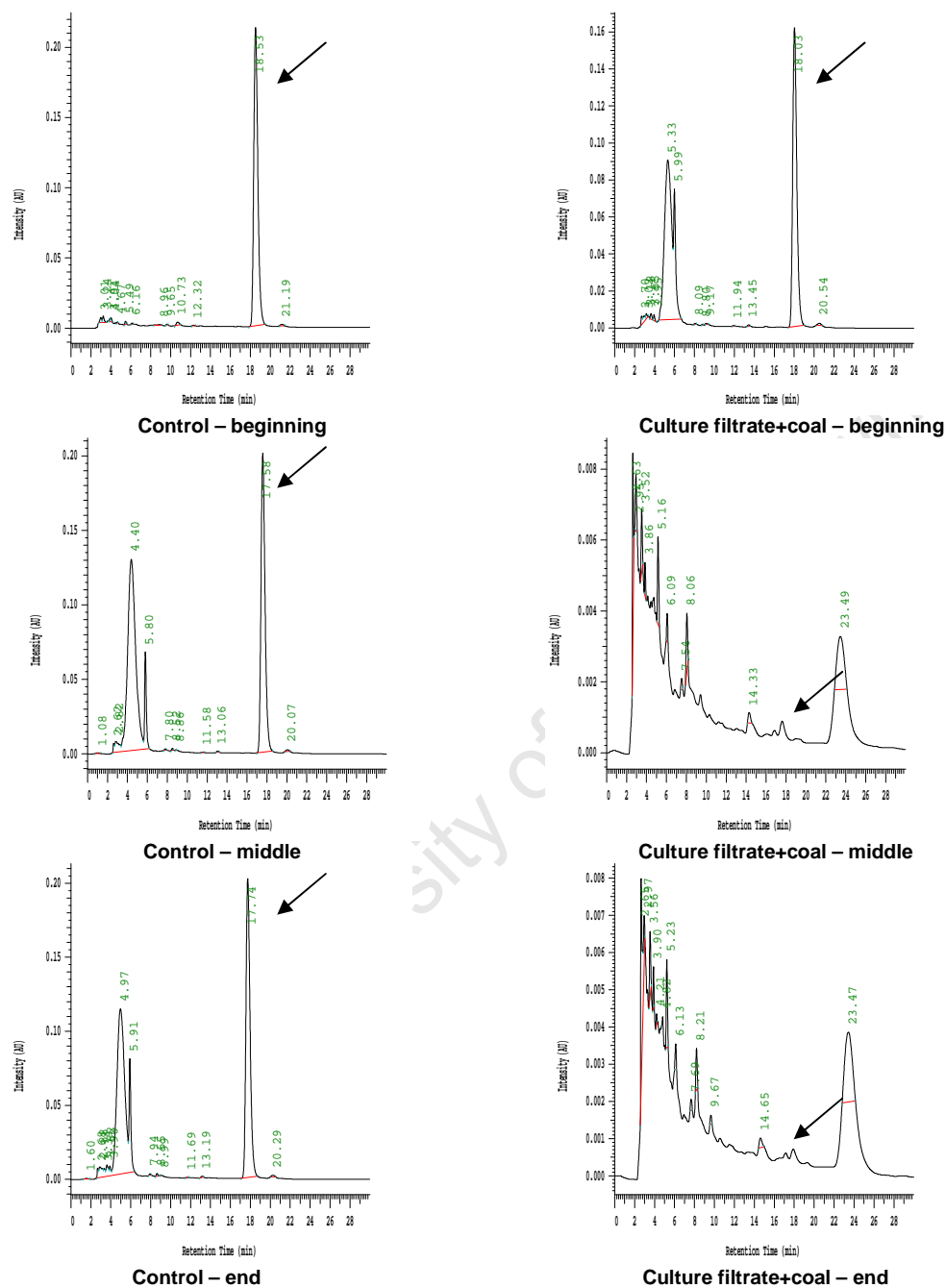


Figure 5.6 Veratryl alcohol measured in the cell-free filtrate from *T. pubescens*, during the bioconversion of sub-bituminous coal, at the start, mid-way and end of reaction (test reaction – right column and control – left hand side), by HPLC. The arrows indicate the peak representing veratryl alcohol.

5.4 CONCLUSIONS

The work presented in this chapter has demonstrated the bioconversion of sub-bituminous coal by a cell-free filtrate from *T. pubescens* exhibiting a high laccase activity. The bioconversion of coal involved depolymerisation reactions, resulting in the production of humic acids and other products of low molecular mass, confirmed by size exclusion chromatography. Quantitative data was provided for bioconversion of coal carbon, as well as the amount of humic acids produced. 97 mg per g of coal carbon was lost due to the bioconversion by the cell-free filtrate from *T. pubescens*, but only 8 mg of humic acids were produced per gram of coal. The small amount of humic acids recovered, in comparison to the amount of coal carbon that was lost, led to the conclusion that the humic acids and other large macromolecules produced from the depolymerisation reaction were further converted. Although a non-enzymatic mechanism(s) was implicated from the results of the control experiment, its effects on coal bioconversion were nominal compared with the action of laccase; this was confirmed by a 9.7% reduction in the carbon content of the coal recovered after treatment with the cell-free filtrate, as compared with only 0.8% reduction found in the control sample.

Similar percentages of coal bioconversion were obtained from the treatment of sub-bituminous coal with the cell-free extract from *T. pubescens* (9.7%), compared to that obtained from coal bioconversion in shake flask cultures of *T. pubescens* (9.5%). In addition, a comparison of the humic acid production during the cell-free treatment of sub-bituminous coal with that produced during the bioconversion of sub-bituminous coal by shake flask cultures of *T. pubescens*, showed that twice as much humic acid was produced in the absence of fungal hyphae. It was therefore concluded that the physical interaction of the coal particles with fungal hyphae (or merely the presence of active fungal mycelia) did not have an effect on the overall coal bioconversion but that it had an effect on the extent of humic acid mineralisation and thus, the fate of coal carbon.

Other than humic acids, 5 products ranging in molecular mass from approximately 530 kDa to less than 60 kDa were detected, by investigation of the molecular mass distribution of the bioconverted product from the treatment of sub-bituminous coal with the cell-free filtrate from *T. pubescens*. Only a single product (of non-enzymatic reaction) was observed in the control, confirming that the bioconversion of coal involved depolymerisation reactions by laccase, resulting in the production of compounds of lower molecular mass. However, these products were much larger than the aromatic monomers which were predicted as products from the cell-free treatment of sub-bituminous coal. Thus, further optimisation of the reaction of the cell-free filtrate from *T. pubescens* with sub-bituminous coal must be carried out in order to obtain the desired aromatic monomers. This would include the optimisation of the reaction time, allowing for optimal depolymerisation, and also ensuring that products are recovered prior to further conversion, that leads to mineralisation.

Veratryl alcohol, initially added as an inducer for laccase and still present upon harvesting the cell-free filtrate, may have played a role as a mediator for laccase in the depolymerisation of coal macromolecules. This theory should have been tested by monitoring coal bioconversion by a cell-free filtrate from *T. pubescens*, that was prepared without the addition of veratryl alcohol, and is recommended for future work.

This is the first report on the bioconversion of untreated sub-bituminous coal by a cell-free laccase-based enzyme extract from *T. pubescens*, resulting in depolymerisation of coal macromolecules.

University of Cape Town

CHAPTER 6: BIOREACTOR SYSTEMS AND KINETIC ANALYSIS OF THE BIOCONVERSION OF COAL BY *TRAMETES PUBESCENS*

6.1 INTRODUCTION

Based on the diminishing supply of natural resources, the utilisation of low quality coal for the production of high-value products will be a viable industry in the near future. However, there are many obstacles to be overcome before economic viability can be achieved. Primary amongst these is the need for effective bioreactor design that takes into account biomass concentration, immobilisation techniques and mass transport phenomena (Klein *et al.*, 1999). An understanding of process kinetics is essential for predicting yields, and therefore, for commercial success of the process. Currently, the yields of processes producing microbially solubilised coal and coal catabolites are low. Kinetic analysis of the bioconversion of coal has not been reported, and most results reported in literature are qualitative, indicating only trends or mechanisms but no rates or kinetics (Klein *et al.*, 1999). The aim of the work presented in this section was two-fold. Firstly, suitable bioreactor configurations were investigated for the bioconversion of sub-bituminous coal by *T. pubescens*, based on knowledge of its growth characteristics and laccase production. Secondly, kinetic analysis of coal bioconversion by *T. pubescens* was performed using the most suitable bioreactor configuration. This section also consolidates the results described previously, and shows the feasibility of using *T. pubescens* in the bioconversion of SA sub-bituminous coal in a bioprocess to produce valuable products. Thus, the work described here is the culmination of this project.

6.1.1 Coal bioconversion in reactor systems

Since biosolubilisation reactions performed in Petri dishes or Erlenmeyer flasks yield only small amounts of solubilised product for chemical analysis or investigation for further biotransformation reactions, larger bioreactors are needed. To date, the study of coal bioconversion in reactor systems has been limited (Hölker and Höfer, 2002). Scott (1990) proposed some basic principles that should be considered when developing advanced bioreactor systems for coal bioconversion. A bioprocess system involving the bioconversion of coal to useful liquid and/or gaseous products includes 4 distinct interacting phases: i) a particulate phase comprising coal particles, ii) biomass, iii) a liquid phase containing nutrients and soluble products, and iv) a gaseous phase containing oxygen as a substrate or carbon dioxide as a product or by-product (Scott, 1990). Ideally, the bioreactor system should operate continuously with optimised mass transfer between the interacting phases. Conventional stirred tank reactors have been modified to achieve continuous operation, but columnar configurations are considered to be more effective for reasons discussed below.

6.1.1.1 Stirred tank bioreactors for coal bioconversion

Stirred tank reactors (STRs) are the most commonly used reactors in bioprocessing. In the STRs used for coal bioconversion, the liquid forms a continuous phase while gas and the heterogeneous coal substrate exist as dispersed phases that are contacted with the suspending fluid. The STR is well mixed by an impeller and/or by the motion of rising bubbles. The energy inputs required for STRs are usually high, and although they have a wide range of applications they are not without limitations. For instance, high solids loading above a certain load (~20%) may lead to reduction in mass transfer and confer stress on the microbial culture (Bailey and Hansford, 1993; Nemati and Harrison, 2000). STRs provide effective oxygen mass transfer and effective mixing but the mechanical agitation can be damaging to microbial cells. This is particularly relevant for filamentous organisms such as fungi, where shear stress has been found to suppress the production of desirable enzymes and metabolites. In addition, growth may be uncontrolled due to a high oxygen concentration and a well-mixed liquid medium rich in nutrients (Couto and Toca-Herrera, 2007).

6.1.1.2 Packed-bed bioreactors for coal bioconversion

One way of controlling the growth rate of a microorganism is through immobilisation. This can be easily achieved in a packed-bed bioreactor, due to the natural tendency of microorganisms to adhere to surfaces (Couto and Toca-Herrera, 2007). In coal biosolubilisation processes, coal acts as the substrate and the immobilisation support. A bed of stationary particles of coal is exposed to the biocatalyst in humid air in a packed-bed bioreactor. Fresh biocatalyst may be added with growth medium or reactant solution at the top of the column, and be allowed to trickle through the bed of coal. The liquid product is collected at the bottom of the reactor, while fresh coal is added at the top (Scott, 1990). Immobilisation systems improve downstream processes since cells are retained in the reactor; this also enables continuous operation. A packed-bed bioreactor system is better suited for processes requiring long retention times, but the success of the operation may be hindered by inefficient transport of liquid nutrient through the bed due to the build-up of biomass (Stitt, 2002).

6.1.1.3 Fluidised-bed bioreactors for coal bioconversion

Essentially, a fluidised-bed bioreactor system for coal biosolubilisation consists of small coal particles suspended in an up-flowing liquid stream containing the biocatalyst. Air is supplied at the bottom of the reactor to maintain good aeration, while fresh coal can be added to the top of the column (Scott, 1990). The liquid product may be collected as a top or side stream from the reactor. Like packed-bed bioreactors, low capital cost, simple mechanical configuration and reduced operating cost due to lower energy requirements make fluidised-bed reactors an attractive alternative to conventional STRs (Bailey and Ollis, 1986). Mass transfer is good and extensive gas evolution is achieved, since suspended particles inhibit gas channelling (Scott, 1990).

6.1.1.4 Airlift bioreactors for coal bioconversion

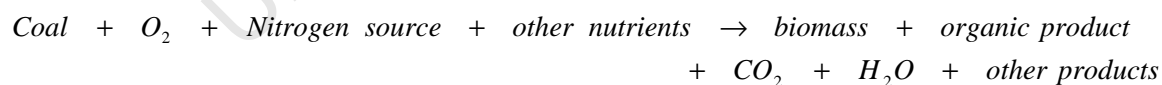
An alternative to the fluidised-bed bioreactor is the airlift reactor, in which an internal or external loop is used to provide fluid circulation. The reactor contains a liquid pool that is divided by a draught tube into two zones, the riser (which is sparged by gas and thus contains the gas-liquid upflow) and the downcomer (containing the down-flowing liquid). Shear forces are evenly distributed throughout the reactor by the draught tube, and are low. Circulation enhances mixing and thus mass transfer in the vessel, which is advantageous for large-scale microbial cultivation (Bailey and Ollis, 1986). Airlift reactors are suitable for the cultivation of filamentous organisms due to the low shear environment they provide. They are characterised by well-defined flow patterns and high liquid velocities despite the absence of a mechanical agitator (Klein *et al.*, 2002). These reactors are now more commonly used in fungal bioprocessing e.g. production of laccase and other oxidative enzymes, which are sensitive to shear stress (Ryan *et al.*, 2005; Rodriguez Couto *et al.*, 2006).

6.1.2 Kinetics of coal bioconversion

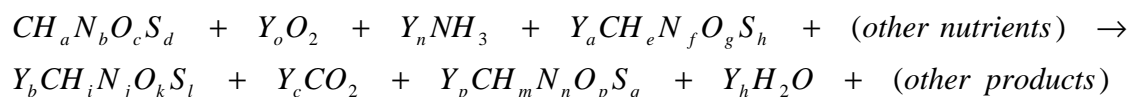
The first step in the development of a bioprocess is a systematic stoichiometric analysis (Bapat *et al.*, 2006). Stoichiometric analysis of a process has proved to be an effective tool for describing the cultivation processes involving growth, medium optimisation and design of feeding strategies (Roels, 1980). Andrews (1991) proposed the application of a standard theory of bioprocess yields to predict yields for coal bioprocesses: mass and energy balances.

6.1.2.1 Evaluation of bioprocess yields using mass balance equations

The first step in constructing a mass balance is to write a pseudo-chemical reaction. For a coal bioconversion process, this would be as follows:



or



.....Equation 6.1

Equation 6.1 is written in terms of carbon equivalents i.e. the amount of organic matter that contains 1 mol of carbon. The Y-values are yields expressed as carbon equivalents, or moles of compounds produced or consumed per carbon equivalent of fuel (Andrews, 1991). The amount and type of product that can be

produced by any coal bioconversion process is limited by the requirements of stoichiometry and energy conservation. Also, the number of available electrons per carbon equivalent in the organic fraction of coal, also known as the degree of reductance, is a critical parameter.

Stoichiometric analysis can be challenging for culturing of microorganisms that involve complex media, due to the ill-defined nature of the medium. Ammonia is shown as a reactant in this generic equation, but more complex nitrogen sources may be used, and these can then be represented as “other nutrients” ($Y_aCH_eN_fO_gS_h$). Water, though represented as a product, may be a reactant if the reaction involves more hydrolysis than dehydration steps. The values of ($a-q$) may be determined through elemental analysis of each reactant and product, and the yield coefficients (Y) may be determined from the five element balances (C, H, N, O, S) and one degree of reductance balance. The degree-of-reduction represents the electron content of a compound relative to the species liberated in a redox half-reaction.

Determining the yield of the process *i.e.* the amount of product that can be obtained from a unit mass of coal, is an important factor that allows for the prediction of the economic feasibility of coal bioconversion (Andrews, 1991). The rate and extent of coal biosolubilisation can be measured spectrophotometrically by an increase in absorbance at 450 nm (Cohen *et al.*, 1987; Hölker *et al.*, 1995), or chromatographically by a decrease in the average molecular mass of coal, or through the production of products such as biomass, carbon dioxide, and phenolic compounds etc. (Ralph and Catcheside, 1997). The most accurate method of determining the extent of coal biosolubilisation is by gravimetric analysis of coal before and after biological treatment. However, this is hindered by the adherence of microbial cells to coal particles, and conventional ways of measuring biomass such as dry weight determinations by filtration or centrifugation in the presence of solid coal particles are not feasible. Also, this method does not allow continuous measurement as is required for determining kinetic yields. Thus, an indirect method of biomass determination needs to be used to overcome this problem. An estimate of biomass production can be obtained based on the stoichiometric relationship between biomass synthesis, oxygen consumption and carbon dioxide evolution, using mass balances. The stoichiometric analysis of bioconversion of coal by a white-rot fungus has not been previously reported.

6.1.2.2 Indirect biomass determination

The relationship between growth and substrate consumption, and growth and product formation can be established by kinetic analysis using the logistic, and Pirt or Luedeking-Piret equations, respectively (Bapat, 2006; Soto-Cruz, 2002; Ariff, 1997). The logistic equation for growth (described in Bailey and Ollis, 1986) is:

$$\frac{dX}{dt} = \mu X \quad \text{.....Equation 6.2}$$

The Pirt equation for substrate consumption (Pirt, 1975) is:

$$\frac{-dS}{dt} = \left[\frac{1}{Y_{x/s}} \cdot \frac{dX}{dt} \right] + mX \quad \text{.....Equation 6.3}$$

The Luedeking-Piret equation for product formation (Luedeking and Piret, 1959) is:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad \text{.....Equation 6.4}$$

Where

t = time

X = biomass concentration

S = substrate concentration

P = product concentration

$Y_{x/s}$ = biomass/substrate yield

m = maintenance coefficient

μ = specific growth rate

α = growth-associated coefficient for product

β = non-growth associated coefficient for product

In this manner the rate of biomass production can be determined by measuring the carbon dioxide evolution rate (CER) and oxygen utilisation rate (OUR). This has been demonstrated for the growth of filamentous fungi on solid wheat grain by Koutinas *et al.* (2003). For an approach such as this to be used, certain assumptions must be made, namely, constant maintenance energy and constant partitioning of substrate between biomass synthesis and product formation. Hence, the yield coefficients based on

oxygen consumption and carbon dioxide evolution remain constant, and by performing a mass balance, the theoretical ratio of substrates used to products formed can be related.

Four bioreactor configurations, a stirred tank slurry bioreactor, packed- and fluidised-bed bioreactors and an airlift bioreactor were investigated to determine their efficacy for the bioconversion of sub-bituminous coal by *Trametes pubescens*. In extending these investigations, a black box stoichiometric model for the bioconversion of coal (a complex carbon source), using peptone as a complex nitrogen source, was developed. An indirect method of biomass determination was used to evaluate the kinetics and product yield of the coal bioconversion process.

This work was performed in collaboration with Mr F.N. Ngako, Mr H.S. Makhongela and Professor S. Harrison from the Department of Chemical Engineering, University of Cape Town.

6.2 MATERIALS AND METHODS

Reagents were all of analytical or HPLC grade as required, and were obtained from Merck, Fluka or Sigma-Aldrich chemical companies (RSA). Sub-bituminous coal (SASOL, 2001) was not pre-treated other than sterilisation by autoclaving at 120 °C, and size-reduction by dry sieving, where specified.

6.2.1 Culture and growth conditions

Trametes pubescens (CBS 696.94) was maintained on 3% malt extract agar plates at 4°C. *Trametes* Defined Medium (TDM) as described in Appendix A, was used for all reactor configurations.

6.2.2 Inoculum preparation

The inoculum was prepared in Erlenmeyer flasks with a volume to air ratio of 1:4 (including a 10% inoculum homogenate) e.g. a 2L flask contained 315 mL growth medium and 35 mL inoculum homogenate. The homogenate consisted of *T. pubescens* from plate culture, homogenised in 200 mL growth medium. The flasks were incubated at 28°C on an orbital shaker at 170 rpm. 5-day old cultures were used as the inoculum.

6.2.3 STR operation

T. pubescens was grown in a two 2-L stirred tank bioreactors, each containing 1.5 L growth medium. The bioreactors, containing baffles, were seeded with a 10% inoculum, aerated at 2 L.min⁻¹, and were mixed using a Rushton turbine impeller at 170 rpm. A constant temperature was maintained by placing the bioreactors in a water bath at 28° C. Coal particles were fractionated to a particle size greater than 425 µm by dry sieving, and were added to the first bioreactor and second bioreactor at a 2 and 10 % (w/v) loading,

respectively, on day 5 of fungal growth. 10 mL- samples were taken daily prior to the addition of coal, and every second day thereafter. Laccase activity, pH, total organic carbon, total phenolic content, and average coal particle size determinations were measured as described in Section 6.2.7.

6.2.4 Fluidised-bed bioreactor operation

A 500 mm long perspex column, with an internal diameter of 25 mm, was sterilised with sodium hypochloride solution (5%) and rinsed with sterile water in a laminar flow hood. 200 mL of a 5-day old *T. pubescens* culture, cultivated in shake flasks, was mixed with 100g of sterile sub-bituminous coal with a particle size of +425 μm . The mixture of coal and fungus was then added to the column. TDM, containing 0.5% glucose (normally 1% glucose), was agitated and aerated in a separate reservoir at 150 rpm and 2 $\text{L}\cdot\text{min}^{-1}$, respectively. The medium was introduced at the bottom of the column by means of a peristaltic pump with a flow rate of 0.78 $\text{L}\cdot\text{hr}^{-1}$. A 5 μm filter was positioned at the base of the column to prevent coal and fungal hyphae from washing out the column. Samples were taken periodically from sample ports positioned on the sides of the column. A diagram of the experimental set-up is shown in Figure 6.1.

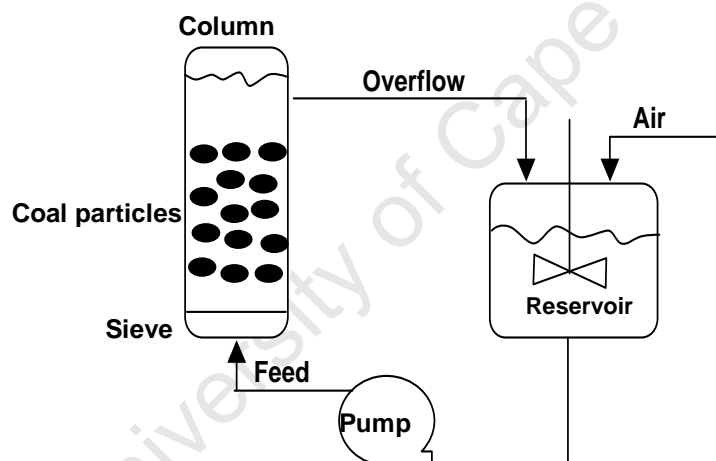


Figure 6.1 Schematic diagram of fluidised bed bioreactor system used for the bioconversion of sub-bituminous coal by *T. pubescens*

In a second experiment, the fungal culture was cultivated in the reservoir for 5 days and the supernatant from this was used to fluidise 50 g of sterile coal (+425 μm). The coal was held in position by sieves with a pore size of 5 μm . Fungal hyphae were prevented from entering the column by a 5 μm filter, fitted to the inlet. Samples were taken daily and were analysed for laccase activity. At the end of the run, the remaining coal in the column was dried and weighed.

6.2.5 Packed-bed bioreactor operation

A 400 mm long column, with an internal diameter of 50 mm, was packed with a mixture of coal and 200 mL of a 5-day old *T. pubescens* culture, as in the fluidised bed bioreactor experiment. The sterilisation method was the same as that used for the fluidised bed reactor. The coal had a particle size of approximately 2.0 mm and was held in a fixed position by a sieve with a pore size of 5 μm . The growth medium, TDM, containing 0.5% glucose, was agitated and aerated at 150 rpm and 2 L.min⁻¹ prior to being pumped from the reservoir to the top of the column. The medium was allowed to percolate through the stationary bed of coal particles and fungal hyphae (Figure 6.2). The underflow (media outlet) was returned to the reservoir and recycled at a pump speed of 0.4 L.hr⁻¹. Samples of the reactor permeate were taken periodically. In a second, similar experiment using the packed bed bioreactor, *T. pubescens* was cultivated in the reservoir for 5 days prior to the commencement of the run. The column was loaded with 50 g of sterile coal, and the percolate consisted only of the supernatant from the reservoir, which was pumped in through the top of the column. Samples were taken daily and were analysed for laccase activity. At the end of the run, the remaining coal in the column was dried and weighed.

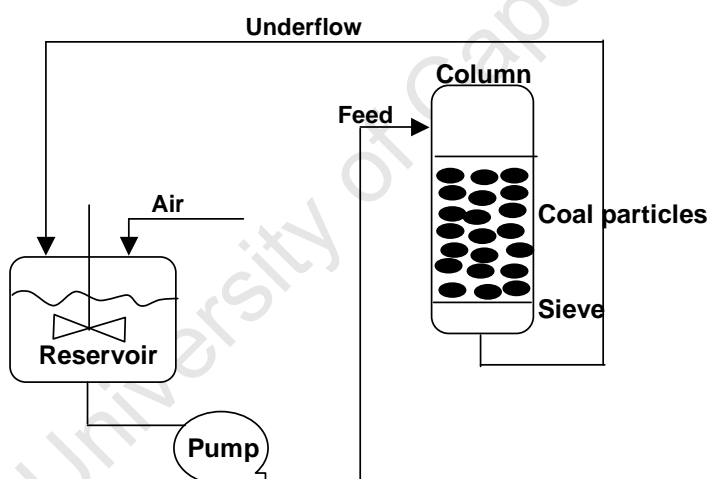


Figure 6.2 Schematic diagram of the packed-bed bioreactor system used for the bioconversion of sub-bituminous coal by *T. pubescens*

6.2.6 ALR operation

The draught-tube internal loop airlift reactor had a total volume of 4 L. The total height was 536 mm, with an internal diameter of 100 mm. The draught tube had an internal diameter of 40 mm, with a riser to downcomer ratio of 1: 5.2. The oxygen sparger was made from sintered steel and can be considered to be a fine bubble diffuser. The vessel and draught tube were made of perspex and could therefore not be autoclaved. Sterilisation was achieved by filling the vessel and all tubing with 10% (v/v) bleach solution and allowing circulation in the vessel for 24 hours. The vessel was subsequently rinsed with sterile, distilled water for 24 hours before the growth medium was added. A peristaltic pump was used to feed the bleach solution, sterile water, growth medium and inoculum into the reactor. Airflow, supplied by a compressor was filter sterilised (Millipore 0.45 μm) and humidified before introduction into the reactor. The airflow rate was controlled using a needle valve, and was measured using a rotameter.

The reactor was filled with approximately 3 L growth medium containing 2 mL antifoam 204 (Sigma) and 220 mL (7%) of inoculum was added. The air supply was turned on after inoculation and set to a flow rate of 3 L.min⁻¹. After 5 days, 500 mL distilled water containing 33g coal that was dry sieved to an average particle size of 212 – 300 μm was autoclaved and after cooling, added to the reactor. 25 mL liquid samples were taken at 24 hour-intervals, frozen and later analysed for glucose, biomass by dry weight determination, laccase activity, pH, UV-Visible spectrophotometry and HPLC analysis. Discreet off-gas samples were taken daily from the exhaust air port at the top of the reactor, and were analysed for carbon dioxide and oxygen concentrations using a Hartmann and Braun off-gas analyser.

The experiment was performed over a 14-day period. Mycelia and coal were separated from the supernatant *via* filtration. 100 mL of the supernatant was acidified to pH 1.5 using 6N HCl to precipitate humic acids. The precipitate was obtained by filtration under vacuum, dried overnight at 80°C and weighed. It was then re-dissolved in 10 mL of 0.1 M NaOH solution. The absorbance of the samples was measured at 450 nm and UV-Visible spectrophotometric scans were compared to a humic acid standard profile.

A summary of the operational parameters is shown in Table 6.1 below.

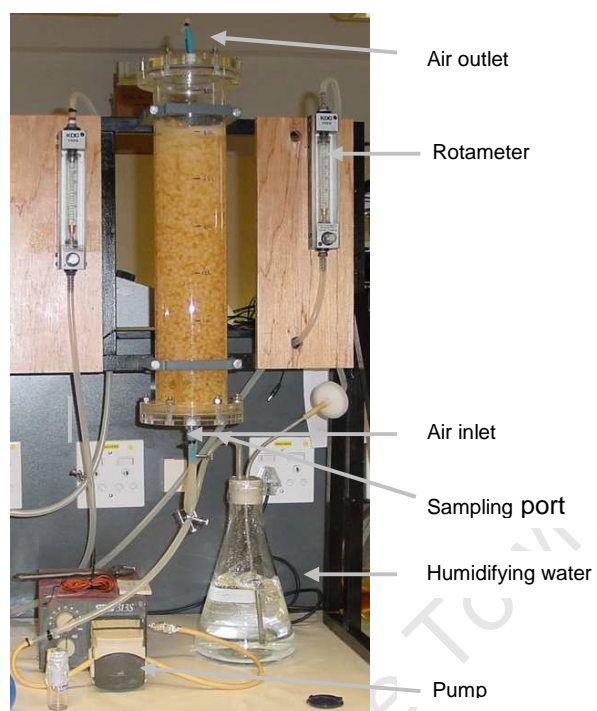


Figure 6.3 Photograph of the draught-tube internal loop airlift bioreactor used for the bioconversion of sub-bituminous coal by *T. pubescens*

Table 6.1 Operational parameters of the airlift reactor system used for the growth of *T. pubescens* with sub-bituminous coal

Condition/Parameter	
Vessel Material	Perspex
Vessel height (mm)	536
Vessel internal diameter (mm)	100
Draught-tube material	Perspex
Draught-tube internal diameter (mm)	40
Sparger	Fine bubble diffuser
Oxygen source	Compressed air (2 bar)
Working volume	3.5 L
Humidification of air	Yes
Sterilisation method	Washed with Bleach solution
Coal particle size (μm)	212-300
Off-gas analysis	Yes

6.2.7 Analytical procedures

6.2.7.1 Glucose assay

The concentration of glucose in the culture filtrate was measured using the reducing sugar assay according to Miller (1959), as described in section 4.2.6.

6.2.7.2 Laccase activity assay

Laccase activity was measured according to Roy-Arcand and Archibald (1991), as described in section 4.2.6.

6.2.7.3 Total phenolic content assay

The total phenolic content of the culture filtrate was measured according to Garcia *et al.* (2001, adapted from Box, 1983) as described in section 5.2.6.4.

6.2.7.4 UV-Visible spectrometry and HPLC analysis

UV-Visible spectrometry and HPLC analysis were performed as described in section 5.2.6.

6.2.7.5 Humic acid precipitation

Humic acids were precipitated from the culture filtrate through the addition of HCl as described in section 5.2.4.

6.2.7.6 Total organic carbon content

Total organic carbon was quantified using an Anatoc Series II TOC analyser. This analysis is based on the oxidation of organic carbon in the supernatant to yield carbon dioxide, which is quantified. Titanium oxide catalyses the oxidation of organic compounds in the presence of UV light and oxygen. Water and carbon dioxide are products of this reaction. This procedure was conducted by the Minerals Processing Research Unit at the University of Cape Town.

6.2.7.7 Average coal particle size analysis

The average coal particle size was determined visually using scanning electron microscopy. The coal particles were fixed onto the slide using graphite and glue. This procedure was performed by the Electron Microscopy Unit in the Physical Science Department at the University of Cape Town.

6.2.7.8 Off-gas analysis and indirect biomass estimation

The carbon dioxide concentration of the off-gas was determined using a Hartmann & Braun Uras 4 NDIR (non-dispersive infrared) industrial photometer and the oxygen concentration was determined using a Hartmann & Braun Magnos 6 G oxygen analyser. Measurement of carbon dioxide and oxygen were used to determine the carbon dioxide evolution rate (CER) and oxygen utilisation rate (OUR) as a means to estimate the rate of biomass production. Growth yield coefficients for *T. pubescens* based on CER and OUR as well as maintenance coefficients were calculated from growth on glucose. The data obtained was used to determine the biomass concentration as a function of time for growth on coal (See Appendix C.6 for sample calculations).

6.3 RESULTS AND DISCUSSION

A preliminary investigation of different bioreactors for coal bioconversion by *T. pubescens* was performed in order to: 1) determine the most suitable configuration, 2) improve the yields of bioconversion products from that obtained in shake flask cultures, and 3) to perform a kinetic analysis. Use of a conventional stirred tank reactor was considered a good starting point because *T. pubescens*, a relatively new isolate, has not been cultivated in many different bioreactor configurations. In addition, since *T. pubescens* showed a high tendency to adhere to coal particles, investigation of immobilised bioreactor systems, such as packed-bed and fluidised-bed bioreactors were also logical choices. Further, previous success of cultivating *T. pubescens* in an airlift bioreactor for the bioremediation of a phenolic effluent by co-workers in our research group led to the choice of this bioreactor system for coal bioconversion (Ryan *et al.*, 2005). Direct comparison of all parameters cannot be made between these configurations since the operating variables were not the same e.g. the particle size of coal, but data presented here may be used to identify trends, and for comparing the economic viability of the processes based on degree of bioconversion, energy input and upstream/downstream processing requirements.

6.3.1 Coal bioconversion by *T. pubescens* in a stirred tank bioreactor (STR)

In the first bioreactor studies, the bioconversion of sub-bituminous coal by *T. pubescens* was carried out in two stirred tank reactors. *T. pubescens* was grown on the defined medium used previously, containing glucose, and coal of a particle size greater than 425 μm . This was added to each bioreactor at 2 or 10% (w/v) loading, on the 5th day of growth. The experiment was performed at a low mixing speed of 160 rpm, comparable to the speed used in orbital shake flask culture experiments. Coal biosolubilisation was measured as a decrease in the average particle size of the residual coal, recovered after the bioconversion, by visualisation using scanning electron microscopy. The laccase activity, pH, soluble organic carbon (total organic carbon) and the total phenolic content in the supernatant of samples, was taken every 48 hours and measured.

6.3.1.1 The effect of solids loading on coal biosolubilisation by *T. pubescens* in a stirred tank bioreactor (STR)

The coal biosolubilisation in the STR experiments was measured as a decrease in average particle size of coal, monitored by scanning electron microscopy. It should be noted that this method was tedious and large variations were associated, due to technical and manipulative difficulties. However, it did provide a qualitative indication of coal biosolubilisation. The average particle size of coal decreased from 837 μm to 476.5 μm , and 577.5 μm , at 10 and 2% coal loading, equating to 43% and 31% decrease in average particle size of coal, respectively (Figure 6.4). A rapid decrease in average particle size occurred between day 0 and 16 of the experiment, at the same rate in the bioreactors containing 2 and 10% (w/v) coal. Thereafter, the rate became steadier, but was higher at 10% than at 2% loading. At the higher coal

loading, a larger surface area was available for coal biosolubilisation to take place but one would also expect a higher degree of inhibition due to the increased hydrodynamic stress. In an experiment done by Oboirien *et al.* (2007) using *Trichoderma atroviride*, a higher conversion was obtained at a 5% coal loading than at 10% loading in shake flask cultures, quantified in terms of the decrease in dry mass (18 and 14% coal weight loss, respectively). This led to the conclusion that the optimum coal loading would lie between 5 and 10%, but additional experimentation would be necessary to confirm this hypothesis. In contrast, Gokay *et al.* (2001) showed that coal biosolubilisation efficiency (based on increase in absorbance at 450 nm) of *Phanerochaete chrysosporium* increased when lignite loading was increased from 5% to 10% (w/v).

In the control experiment, the same conditions were applied except that no fungal culture was present. A decrease from 837 μm to 774 μm was obtained, indicating a 7.5% reduction in the average coal particle size attributable to attrition. Since no fungal hyphae were present in the control experiment, the solubilisation of coal could also be measured gravimetrically. In this case, a loss of 10.45 g of coal was measured, indicating a 7% loss in coal weight due to attrition; this value was close to the value obtained using average particle size analysis, which validated the size analysis method.

These results showed that the overall rate of coal biosolubilisation was higher at 10% coal loading than at 2% loading. It was also notable here that the decrease in average coal particle size coincided with an increase in laccase activity over time (see Figure 6.5 and Section 6.3.1.2 below).

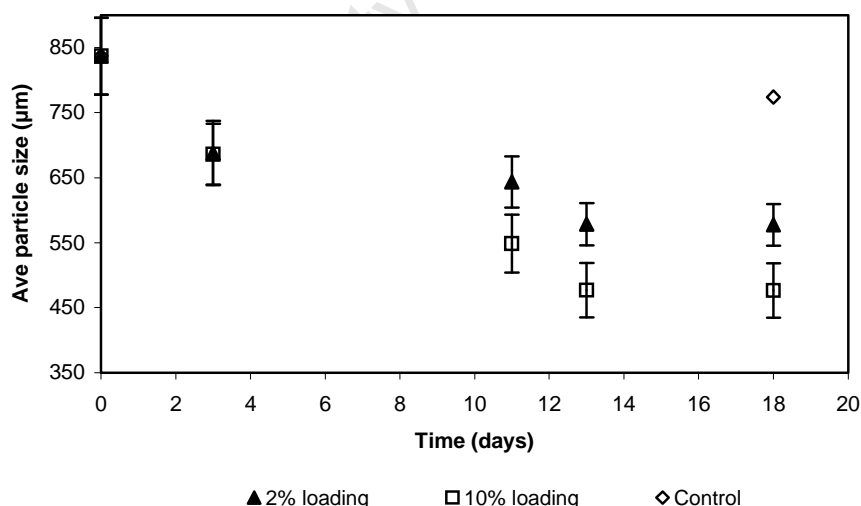


Figure 6.4 Decrease in the average particle size of coal during its bioconversion by *T. pubescens* in stirred tank bioreactors at 2 or 10% (w/v) coal loading, respectively (The control bioreactor contained coal and growth medium, in the absence of the fungus.)

6.3.1.2 The effect of solids loading on laccase activity of *T. pubescens* in a STR

The profiles of laccase activity obtained from the STR experiments with 2% and 10% (w/v) coal loading were similar (Figure 6.5). Maximum activities of 4.3 U mL^{-1} and 3.3 U mL^{-1} were obtained at 2% and 10% coal loading, respectively, after 2 weeks of growth. One might have expected a much lower activity at 10% coal loading, since previous work in shake flask cultures, and other reports in literature, have shown laccase to be sensitive to the presence of coal particles. A decrease in laccase activity produced by *T. versicolor* in shake flasks from 0.37 U mL^{-1} to 0.016 U mL^{-1} was measured when 0.05% (w/v) lignite was added to the culture (Fakoussa and Frost, 1999). This lignite concentration was 200-fold lower than the highest concentration of coal used in the current study, suggesting that the laccase produced by *T. pubescens* was not sensitive to high solids loading.

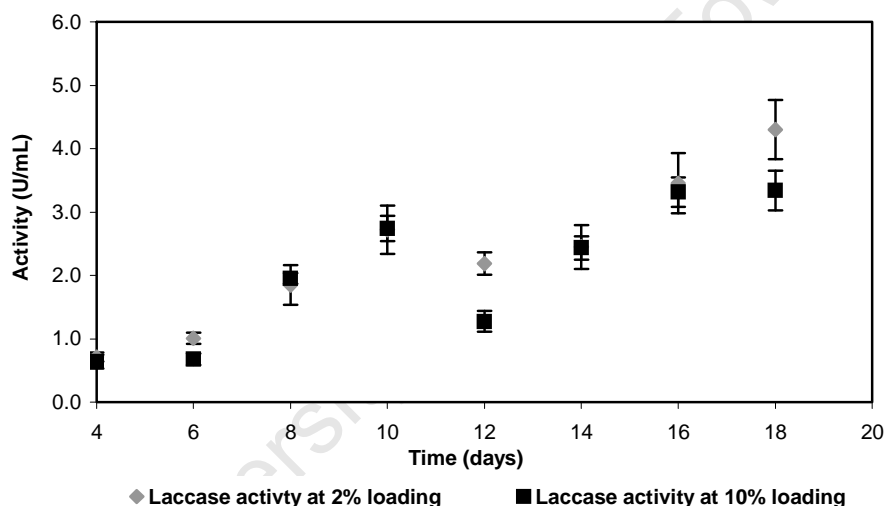


Figure 6.5 Laccase activity of *T. pubescens* during bioconversion of sub-bituminous coal in stirred tank bioreactors at 2 or 10% (w/v) coal loading (Coal was added on the 5th day of growth.)

The laccase activity measured in this experiment increased after the addition of coal, from approximately 0.7 U mL^{-1} to 4.2 U mL^{-1} and 3.4 U mL^{-1} in the bioreactors with 2% and 10% (w/v) coal, respectively. However, a decrease in laccase activity, from 4 U mL^{-1} to 2.8 U mL^{-1} , was observed after the addition of coal in shake flask culture experiments, where 1% (w/v) coal loading was used (see section 4.3.2.2). The increase in activity in the STRs may be attributed to the increased aeration since air was introduced at a flow-rate of $2 \text{ L} \cdot \text{min}^{-1}$ (1 vvm). Oxygen transfer is often a rate-limiting step in aerobic bioprocesses due to the low solubility of oxygen in water. The oxygen demand is a function of the culture growth phase, biomass production or product yield (Bailey and Ollis, 1986). In terms of laccase production by *Panus tigrinus* in an STR, Fenice *et al.* (2003) showed that aeration rate affected the enzyme activity to a large

extent and that the highest laccase activity produced was obtained at 1 vvm, whereas lower activities were obtained at 0.5 and 1.5 vvm.

These results from the STR experiment, together with the shake flask culture experiments, led to the conclusion that the laccase activity produced by *T. pubescens* in a slurry system was affected more by the amount of available oxygen than by presence of solid coal particles.

6.1.1.3 The effect of solids loading on pH, total organic carbon and total phenolic content of supernatant from the bioconversion of coal by *T. pubescens* in the STRs.

In the coal bioconversion experiments conducted with *T. pubescens* in STRs with 2% and 10% (w/v) coal loading, the pH, total organic carbon and total phenolic content profiles, obtained at different loadings, showed an negligible differences. An overall increase in pH from 4.86 to approximately 7 was observed in both bioreactors, indicating that the change in pH was independent of coal loading (Figure 6.6). In a similar study using lignite coal pre-treated with nitric acid, Quigley *et al.* (1988) reported a correlation between pH and coal biosolubilisation, claiming that the pH of the medium increased due to the production of alkaline substances involved in the biosolubilisation of coal. However, Laborda *et al.* (1997) reported that the variation in pH did not correspond to coal biosolubilisation, and that the relationship between pH and coal biosolubilisation was irrelevant when untreated coals were used; this is in better agreement with results obtained in the present study. In additional support of this, results presented later in this chapter (Section 6.33, Figure 6.12) showed an increase in pH, when *T. pubescens* was grown in an airlift reactor, both in the presence and absence of coal, showing that the production of alkaline substances did not occur in response to the presence of coal.

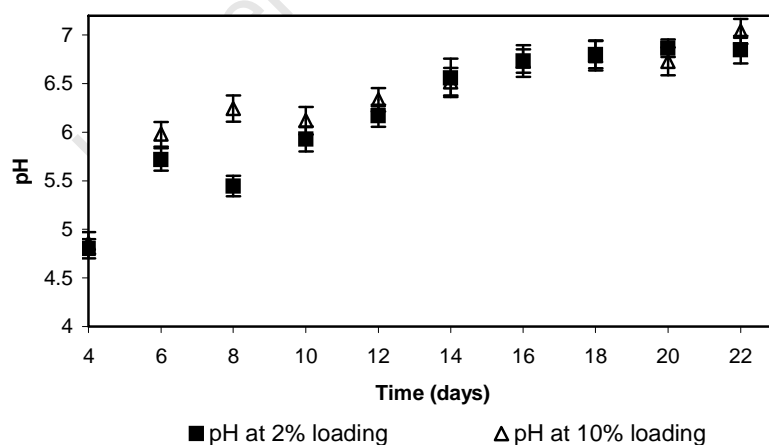


Figure 6.6 The pH profile of the supernatant from *T. pubescens* grown in stirred tank bioreactors at 2 or 10% coal loading

The total organic carbon (TOC) is a measure of the soluble carbon content of a solution after filtration, and was therefore used as a means of quantifying soluble organic compounds released into the supernatant, during the bioconversion of sub-bituminous coal by *T. pubescens* in the STRs. At the beginning of the cultivation, any changes in the TOC could be attributed to the uptake of glucose by the fungus. As coal became degraded and products were released, the TOC was expected to increase. However, this was not observed. Furthermore, negligible differences in the TOC content of the supernatant were evident between samples collected from the STRs containing 2% or 10% coal (w/v). The possible explanation for this is that the products were taken up by the fungus and metabolised as the coal was being degraded, to form carbon dioxide. Alternatively, the sampling might not have been frequent enough to allow observation of changes in the TOC before the products were taken up by *T. pubescens*. Only continuous online monitoring would allow this, but was not possible during this project.

Similarly, an increase in the phenolic content of the supernatant was expected as a result of coal degradation in the STRs. The release of phenols and polyphenols was expected based on the structure of coal, and reactions catalysed by laccase. However, only very minor changes in the total phenolic profiles were observed and were attributed to uptake of growth medium constituents such as veratryl alcohol, which was added as an inducer of laccase. The explanations proposed for the unchanging TOC values, that the products were taken up by the fungus as the coal was being degraded and metabolised to form carbon dioxide, would also be applicable to the unchanging concentration of phenolics in the supernatant.

Stirred tank bioreactors are not commonly used for bioprocesses involving filamentous fungi, mainly due to the damaging shear effects of mechanical agitation. However, STRs are characterised by good mass transfer coefficients and oxygen transfer rate. They are well mixed and good suspension is achievable. The results presented here are preliminary, but have demonstrated the feasibility of using a stirred tank slurry bioreactor for the bioconversion of coal at solid loadings that might have been expected be inhibitory. In terms of economic feasibility and scaling up of the process, additional experimentation to investigate numerous parameters such as optimum coal particle size, aeration rate, agitation etc, would be required. Further, one of the major drawbacks of STRs is the high energy input requirement associated with scale-up. The addition of milling costs, should milling be required to obtain an optimal coal particle size, might provide the incentive for choosing an alternative reactor configuration, such as those discussed below.

6.3.2 Coal bioconversion by *T. pubescens* in fluidised- and packed-bed bioreactors

The bioconversion of sub-bituminous coal by *T. pubescens* was investigated in fluidised and packed-bed bioreactors because these configurations require a solid matrix as an immobilisation support for microorganisms, and since coal is solid substrate, it could also be used as the immobilisation support. Further motivation for using these bioreactors was the attachment of fungal hyphae to coal particles, observed in shake flask culture and STR experiments.

The fluidised-bed bioreactor experiments were carried out in two ways. The first experiment entailed the mixture of coal particles (425 μm) with a 5-day old *T. pubescens* culture, prior to addition to the column. Fluidisation was achieved by pumping growth medium (that had been aerated in a separate reservoir) containing 0.5% glucose (reduced from the 1% glucose concentration used in the STRs) into the reactor from below. The glucose content of the growth medium was reduced to half its original concentration to avoid excessive growth that was expected in the bioreactor, and to stimulate an increase in laccase production. Glucose, when present in cultures at a high concentration, is considered to repress laccase synthesis in *T. pubescens*. This catabolite repression, known in other fungi and yeasts, is thought to be an energy saving response (Galhaup *et al.*, 2002). Despite this lower glucose supply, fungal growth inside the column of the fluidised-bed bioreactor caused the bed to move upwards, and this resulted in wash-out of coal particles and fungal hyphae.

The second experiment with the fluidised-bed bioreactor was undertaken in response to the failure of the first; here similar conditions were used as described for the experiment, except that *T. pubescens* was cultivated in a reservoir and when the culture was 5 days old, only the supernatant was used to fluidise the coal particles. The experiment continued successfully for 6 days until the 5- μm filter, fitted to the inlet pipe, to prevent fungal hyphae from entering the column, became blocked by fungal growth. The total phenolic content of the supernatant measured on days 4, 5 and 6 did not change, and the laccase activity in the supernatant is shown in Figure 6.8 (discussed later). Since no wash-out occurred in this experiment and the fungal hyphae were prevented from entering the column, coal degradation was measured as a decrease in the dry weight of the residual coal recovered at the end of the experiment. The initial mass of coal added to the column was 50 g, while the mass of coal remaining after treatment with *T. pubescens* culture supernatant was 44.49 g. This equated to an 11% conversion of coal, which was still a good result considering the experimental challenges associated with this bioreactor set-up.

The packed-bed bioreactor experiments were performed in much the same way as was described for the fluidised-bed bioreactor experiments. Initially, a 5-day old culture of *T. pubescens* was mixed with coal particles (2000 μm) prior to addition to the column. Growth medium (containing 0.5% glucose) that was aerated in a separate reservoir was pumped in through the top of the column and allowed to filter through coal particles and fungal pellets. Fungal growth in between coal particles again caused the bed to move

upwards and the void spaces were invaded. As a result, the growth medium could no longer filter through the bed and the system became fouled. The experiment was stopped after 2 days.

In the second experiment, *T. pubescens* was cultivated in a separate reservoir and after 5 days of growth only the culture supernatant was pumped into the column. The bioreactor operated successfully for 5 days until the inlet filter became blocked. Although the experimental time was short, laccase activity in the supernatant (Figure 6.7) and dry weight analysis of residual coal particles were determined. The mass of coal was reduced by 2.5 g after 5 days of treatment with the supernatant from *T. pubescens* in the packed-bed bioreactor, yielding a 4.9% conversion of sub-bituminous coal.

Laccase activity of approximately 1.2 U mL^{-1} was measured in the supernatant circulating in the fluidised-bed reactor compared with a laccase activity of 0.9 U mL^{-1} in the packed-bed reactor. This was an interesting result since the same conditions were applied to each reservoir used to cultivate the *T. pubescens* culture from which the supernatant was harvested. The presence of smaller coal particles (offering an increased surface area), and good mixing and mass transfer typically associated with fluidised-bed bioreactors (Nicolella *et al.*, 1997; Scott, 1990) may have caused this stimulatory effect on laccase activity. Further, and importantly, the higher laccase activity found in the fluidised-bed bioreactor system also correlated with an 11% coal conversion which was higher than that obtained in the packed-bed bioreactor (4.9%) (Table 6.2). This result was comparable with the results obtained from the bioconversion of sub-bituminous coal by the cell-free filtrate from *T. pubescens* (see Section 5.3.3), further highlighting the involvement of laccase in the bioconversion of sub-bituminous coal by *T. pubescens*.

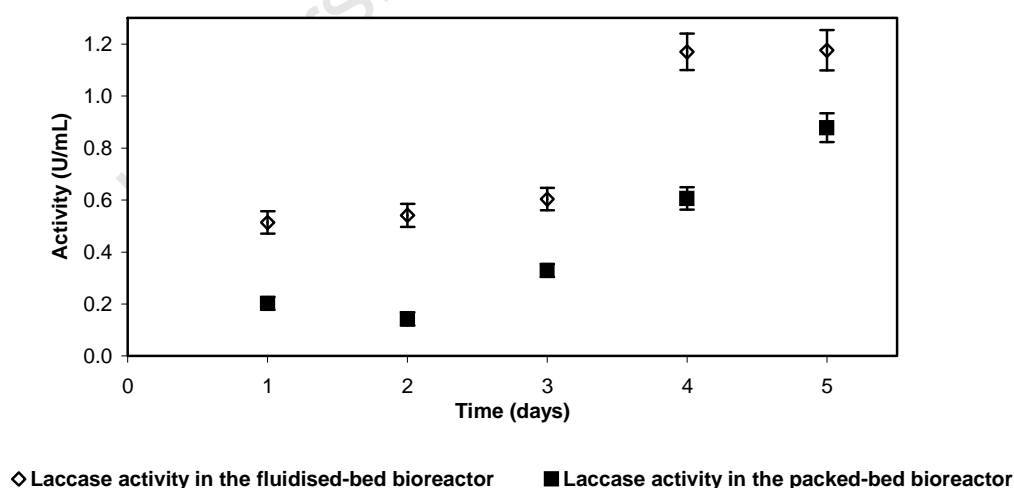


Figure 6.7 Laccase activities in the supernatant (obtained from *T. pubescens* grown in a separate reservoir) which was circulated through the fluidised-bed bioreactor and packed-bed bioreactor

Table 6.2 Relationship between percent coal conversion, determined by dry weight analysis, and laccase activity by *T. pubescens* in fluidised-and fixed-bed bioreactors

	% Coal conversion	Laccase activity*
Fluidised-bed bioreactor	11	1.175
Packed-bed bioreactor	4.9	0.878

* at the end of experiment

Based on the results obtained here, the fluidised-and packed-bed bioreactors used for the bioconversion of sub-bituminous coal were better suited to cell-free preparations of *T. pubescens* rather than cultures including fungal mycelia. Optimisation of these bioreactor configurations, in order to make them more suitable for use in coal bioconversions by a *T. pubescens* culture, could include limiting the nutrient supply in a continuous mode of operation and/or the introduction of gas pulsation to maintain fungal cultures as discrete pellets in a fluidised-bed bioreactor (Moreira *et al.*, 1998; Mielgo *et al.*, 2002), but this was outside the scope of the present study.

6.3.3 Coal bioconversion by *T. pubescens* in an ALR

Airlift reactors have been found to be suitable for the cultivation of filamentous microorganisms due to the low shear but highly oxygenated environment that they provide. The bioconversion of sub-bituminous coal by *T. pubescens* was performed in a draught-tube internal loop airlift reactor due to the simplicity of its operation, and following the difficulties experienced when running the fluidised and packed-bed bioreactors. Sterile coal, with a particle size of 212-300 μm , was added after 5 days of growth, just before the glucose was totally depleted, to allow for the co-metabolic degradation of coal, as required in lignin biodegradation (Kirk and Farrell, 1987). Figure 6.8 shows the profiles of the glucose consumption and biomass production by *T. pubescens* before the addition of coal on the 5th day of growth. The shapes of the curves resemble classical concentration-time profiles for standard batch operation. The biomass concentration reached 3.13 g L⁻¹ after 5 days. Once the coal was added, the biomass concentration could not be determined due to the attachment of fungal hyphae to coal particles, as had been observed in shake flask culture experiments (Section 4.3.2.2). The results of the indirect biomass measurement in the presence of coal are presented later in this chapter (Section 6.3.4.2).

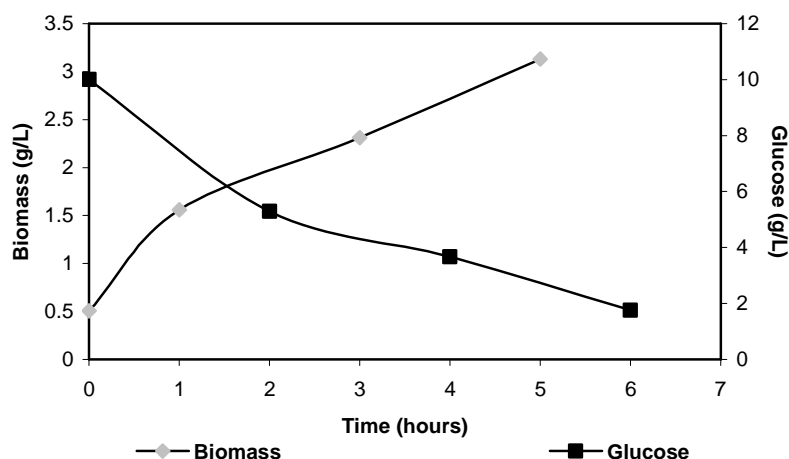


Figure 6.8 Glucose consumption and biomass accumulation during the growth of *T. pubescens* in an airlift bioreactor (Values represent the average of duplicate samples with a standard deviation of less than 10%.)

6.3.3.1 Production of laccase and humic acids during the bioconversion of coal by *T. pubescens* in the ALR

The production of laccase by *T. pubescens* steadily increased during growth with coal in the airlift reactor (Figure 6.9) and a much higher activity was obtained when *T. pubescens* was grown in the presence of coal than in the control where no coal was added. A maximum activity of 8.3 U mL^{-1} was obtained, while only 1.2 U mL^{-1} was obtained in the control culture grown in the absence of coal. The laccase activity in the control culture was notably lower than in the test culture grown in the presence of coal, which may have been due to slight differences in the separate airlift reactor vessels used (e.g. slight variation in the spargers), or the presence of coal could have had an inductive effect on the production (or release) of laccase by *T. pubescens* in the airlift reactor. The high laccase activity obtained in this study is an interesting and significant result considering that the presence of solid substrate particles contributes to shear stress experienced by the fungus, and that an inhibitory effect of coal on laccase activity was established in shake flask culture experiments (see Section 4.3.2.2). However, the presence of solids in a slurry reactor also affects the viscosity, which in turn affects the overall mass transfer coefficient for oxygen (Oguz *et al.*, 1987). Thus, the higher laccase activity produced by *T. pubescens* in the airlift bioreactor, in the presence of coal, may have been a consequence of improved oxygen mass transfer (see Section 6.3.1.2). This could have been tested by monitoring the dissolved oxygen (DO) content in culture using a DO probe, but suitable apparatus was not available during this project.

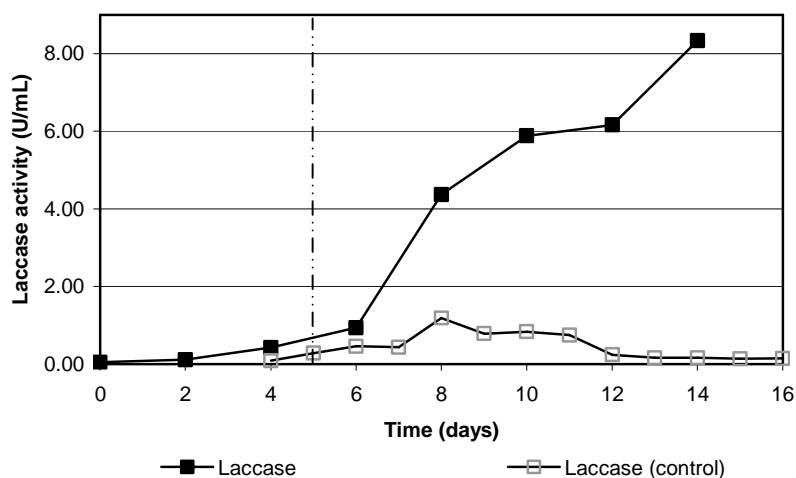


Figure 6.9 Production of laccase by *T. pubescens* grown in the presence and absence of coal in an airlift bioreactor (Coal was added on day 5 (----) Values represent the average of duplicate samples with a standard deviation of less than 5%.)

The steep increase in laccase activity, at day 6, coincided with the production of humic acids, as indicated by the increase in absorbance of the culture filtrate at 450 nm (Figure 6.10), as observed in shake flask culture experiments with *T. pubescens* (see Section 4.3.2). An increase in absorbance was noted in the control experiment, possibly due to the production of melanins (as described in Section 3.3.1) but after the 5th day, the absorbance decreased. Once the experiment was completed, humic acids were extracted from the supernatant by acid precipitation. The precipitated humic acids were re-dissolved in sodium hydroxide solution and the absorbance was again measured at 450 nm, and compared with the absorbance of a pure humic acid standard. 1.07 mg humic acid was produced per gram of coal, by *T. pubescens* in an airlift reactor (Table 6.3). This value was considerably lower than the amount of humic acids (7.6 mg/g coal) produced by cell-free filtrates of *T. pubescens* in shake flask experiments (see Section 5.3.3.2). The attachment of humic acids to fungal mycelia could have occurred, but it was also likely that the humic acids were further degraded and metabolised, resulting in a lower concentration of humic acids remaining when respiring *T. pubescens* mycelia were present. In further support of this theory, the ability of *T. pubescens* to grow on humic acids, as a sole carbon source, was shown in the solid media screening assays described in Section 2.3.2.2.

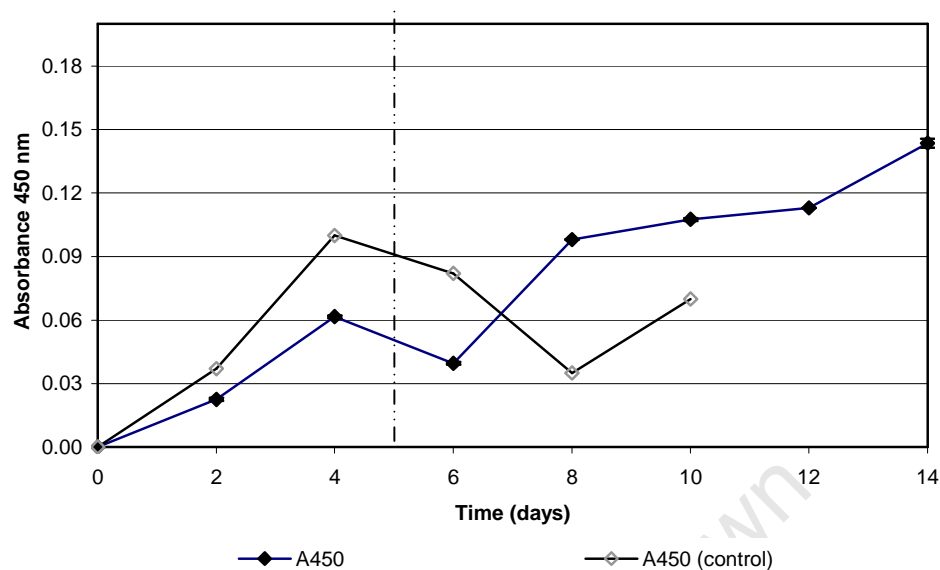


Figure 6.10 Production of humic acids by *T. pubescens* grown in the presence and absence of coal in an airlift reactor (Coal was added on day 5 (----) Values represent the average of duplicate samples with a standard deviation of less than 5%.)

Table 6.3 Relative amounts of humic acids produced from the bioconversion of sub-bituminous coal by *T. pubescens* in an airlift reactor calculated from the absorbance obtained from a pure humic acid standard solution

Sample	Absorbance 450 nm (AU)*	Mass of humic acid (mg) in 10 mL	Mass of humic acid (mg) in 3.5 L	Mass of humic acid/ per mass of coal (mg/g)
Humic acid std (100x dilution)	0.33	51.6	N/A	N/A
Reaction mixture	0.68	1.07	37.45	1.07
Control	0.28	0.44	15.4	0.44

* Values are the average of duplicate samples with a maximum standard deviation of less than 5%.

6.3.3.2 Change in pH of the supernatant during the bioconversion of coal by *T. pubescens* in the ALR

The pH profile of the supernatant from the culture of *T. pubescens* grown in the presence and absence of coal is shown in Figure 6.11 below. A decrease in medium pH from 5 to about 3.6 is characteristic of *T. pubescens* during its growth phase. However, as glucose becomes depleted, the pH steadily increases (Galhaup *et al.*, 2003). Thus, in the present study, a decrease in pH was observed over the first 4 days of growth, and later, as the glucose became depleted, an increase in pH was observed. Similar observations were made in shake flask culture experiments (section 4.3.2.2). Since an increase in pH was also observed in the control experiment, where no coal was present, it was concluded that the production of alkaline substances was not induced by the presence of coal during the bioconversion of sub-bituminous coal by *T. pubescens*.

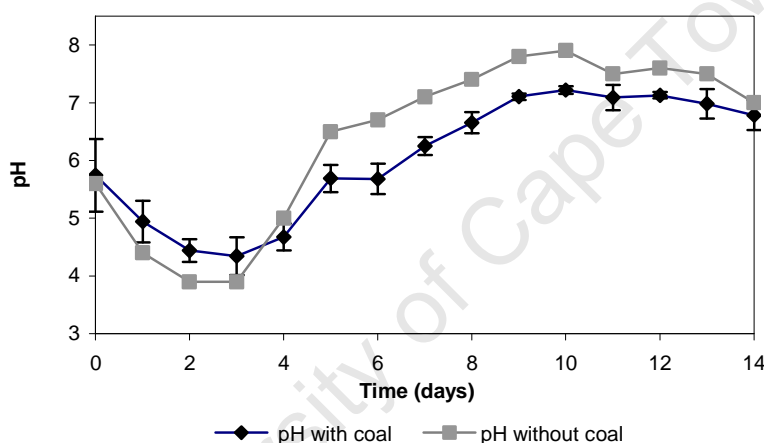


Figure 6.11 The pH profile of supernatant from the *T. pubescens* culture, grown in the presence and absence of coal in an airlift reactor

6.3.3.3 Depolymerisation of coal during its bioconversion by *T. pubescens* in the ALR

The analysis of the molecular mass distribution of products from the bioconversion of sub-bituminous coal by *T. pubescens* in the airlift bioreactor using HPLC-SEC indicated the depolymerisation of polymers to yield compounds of lower molecular mass (Figure 6.12). In a screening assay developed by Hofrichter and Fritsche (1996; 1997a, b), the ability of fungi to depolymerise low rank coal was evidenced by the depolymerisation of coal humic acids of high molecular mass, producing low molecular mass fulvic acids. Thus, in the present study, the depolymerisation of sub-bituminous coal, by a cell-free extract of *T. pubescens*, producing humic acids was shown (see Section 5.3.4), but in the presence of fungal mycelia, the high molecular weight fraction, representing humic acids (approximately 1000 kDa – 530 kDa), was

absent, indicating that the humic acids were depolymerised, by laccase, to form low molecular mass fulvic acids (approximately 20 kDa) (Figure 6.12). This production of lower molecular mass compounds was observed on day 10 (peaks 1-3) of the experiment, but by day 14 these compounds were no longer detected suggesting that mineralisation had occurred. This further supports the hypothesis that mineralisation of smaller compounds, formed from the depolymerisation of coal, occurred due to intracellular uptake and metabolism by *T. pubescens*, which was facilitated by the close physical association between coal particles and fungal mycelia. This phenomenon was demonstrated during the biodegradation of lignin by *P. chrysosporium* (reviewed by Odier and Artaud, 1992).

Furthermore, the depolymerisation of native (non-pretreated) sub-bituminous coal has not been previously reported. In contrast to the results presented here, an increase in the amount of compounds (with a molecular weight of approximately 20 kDa), was noted, during the bioconversion of alkali-solubilised Morwell brown coal by *P. chrysosporium*, suggesting that polymerisation reactions might have occurred (Ralph and Catcheside, 1997). The findings of the current study are therefore important, highlighting the potential of using *T. pubescens* and/or its metabolites in a bioprocess to produce useful low molecular mass compounds from low rank coal. The use of cell-free filtrates from *T. pubescens* may be useful for minimising mineralisation reactions, and thus increasing the yield of low molecular mass compounds, as described in Chapter 5.

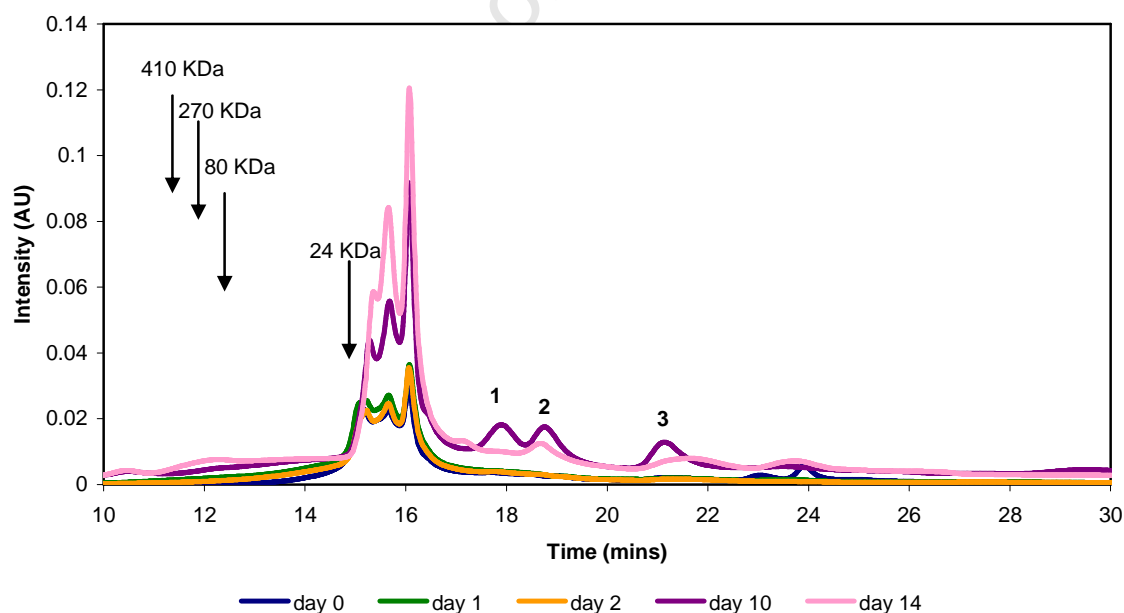


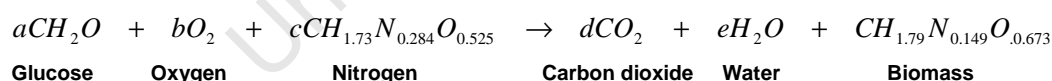
Figure 6.12 Variation with time in the molecular mass distribution of products from the bioconversion of sub-bituminous coal by *T. pubescens* in an ALR

6.3.4 Kinetics of coal bioconversion

The evaluation of coal bioconversion, in terms of process feasibility, is dependent on determination of the product yield. In the present study, this required the measurement of coal consumed, and biomass and other products obtained from the bioconversion by *T. pubescens* in the ALR. The presence of solid coal substrate hindered the measurement of biomass and residual coal, and therefore a stoichiometric analysis of the bioconversion of sub-bituminous coal by *T. pubescens* was performed in order to obtain theoretical process yields. A stoichiometric analysis was performed by using elemental balances and rate measurements obtained from the airlift reactor experiments. Biomass production was measured indirectly using the carbon dioxide evolution rate (CER) and the oxygen utilisation rate (OUR), determined from the carbon dioxide and oxygen concentrations in the off-gas, respectively.

6.3.4.1 Evaluation of *T. pubescens* growth on glucose in an airlift bioreactor

During the cultivation of *T. pubescens* in the presence of coal in the ALR, its growth was first established on glucose, before the addition of coal. The first step in the stoichiometric analysis was, therefore, to evaluate the growth of *T. pubescens* on glucose. *T. pubescens* was grown on a defined medium (TDM), containing glucose and peptone as the carbon and the nitrogen source, respectively, before the addition of coal. The glucose consumption and biomass production by *T. pubescens* in the ALR, before the addition of coal, showed classical concentration-time profiles for a standard batch culture as described in section 6.2.6. The general form of the stoichiometric equation for microbial growth on glucose, based on the assumption that no extracellular organic products were formed, is shown in Equation 6.5 below (adapted from Bailey and Ollis, 1986). The formula for peptone (nitrogen source) was derived from the amino acid composition provided by the manufacturers, while the biomass formula was calculated from the elemental composition of a dried biomass sample from the ALR determined by microanalysis as described in Section 5.2.8 (Appendix C1 and C2).



.....Equation 6.5

The stoichiometric coefficients (*a-e*) were determined by using four element balances and the experimental biomass yield ($Y_{X/S}$) as shown below:

Elemental balances

$$\text{C: } a + c = d + 1$$

$$\text{H: } 2a + 1.73c = 2e + 1.79$$

$$\text{N: } 0.284c = 0.149$$

$$\text{O: } a + 2b + 0.525c = 2d + e + 0.673$$

Charge balance

$$\gamma: 4a - 4b + 3.83c = 4$$

By simplifying the above equations, the following equations were obtained:

$$a = d + 0.475$$

$$b = a - 0.95$$

$$c = 0.525$$

$$d = b + 0.022$$

$$e = a - 0.441$$

The **experimental biomass yield** (Figure 6.8) was used to determine a :

$$\frac{\Delta \text{ biomass}}{\Delta \text{ glucose}} = \frac{2.24 \text{ g}}{8.427 \text{ g}} = \frac{2.24 \text{ g} / 31.87 \text{ g/mol}}{8.427 \text{ g} / 180 \text{ g/mol}} = \frac{0.0703 \text{ mol}}{0.0468 \text{ mol}}$$

= 1.5 mol biomass per mol glucose or 0.67 mol glucose gives 1 mol biomass

Therefore,

$$a = 0.67$$

$$c = 0.53$$

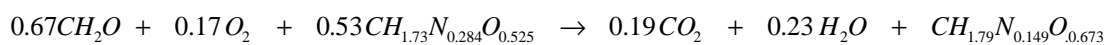
By substituting a and c into the simplified elemental balances,

$$b = 0.17$$

$$d = 0.19$$

$$e = 0.23$$

Thus, the equation for growth on glucose is given in Equation 6.6 below.



.....Equation 6.6

The theoretical yield coefficients based on the stoichiometric equation, given in Equation 6.6, and experimental yields were compared and are represented in Table 6.4. Sample calculations of the experimental yields are shown in Appendix C.4.

The theoretical biomass yield on oxygen (Y_{X/O_2}), based on stoichiometry, was 5.89 g biomass. $g^{-1} O_2$, while the experimental yield based on oxygen utilisation was 0.2 g biomass. $g^{-1} O_2$. This indicates that the growth reaction was less energy-efficient than predicted stoichiometrically, in the absence of extracellular products and substantial maintenance energy. Consideration of the curve of the cumulative oxygen consumption over the 14-day growth period showed that the rate of oxygen consumed was lower during the first 5 days with glucose as the primary growth substrate, than after coal was added on day 5 (Figure 6.13). This makes the accuracy of the oxygen consumption data questionable since one might expect the cumulative oxygen consumed for growth on glucose (a readily utilisable carbon source) to be much greater than that for growth on coal (a more recalcitrant, complex carbon source). The measurement of oxygen concentrations may have been inaccurate due to the small percentage changes in the vent O_2 concentrations, leading to large percent errors (as was discovered by Bapat *et al.* (2006) during the growth of *Amycolatopsis mediterranei* on glucose). Measuring the dissolved oxygen (DO) content on-line using a DO probe is a more accurate alternative for calculating oxygen consumption, but was not possible in the present study and should be considered in future. The contribution of non-biological reactions to oxygen consumption was not considered here, but in order to refine the data for accurate application and to ensure that the system is not oxygen-limited, it should be part of future investigations.

The theoretical yield of carbon dioxide ($Y_{CO_2/X}$) based on stoichiometry was 0.27 g CO_2 . g^{-1} biomass, while the experimental yield based on carbon dioxide production was 2.81 g CO_2 . g^{-1} biomass. This indicates that the growth reaction was more energy-efficient than predicted stoichiometrically in the absence of extracellular products and substantial maintenance energy. The rate of carbon dioxide produced on glucose was higher than that produced on coal, as was expected (Figure 6.13). The carbon dioxide production data appeared to be more accurate than the oxygen consumption data, presumably because the normal average concentration of CO_2 in air is low, and thus, changes in CO_2 concentrations due to microbial respiration are larger and more accurately measured. (In comparison, the O_2 content of air is high, and changes in O_2 concentrations caused due to respiration are small and therefore less accurately measured.) The accuracy of this data may still be improved by measuring the CO_2 concentrations on-line as opposed to taking daily discrete samples, as done in this study.

It is important to note that the data presented on the offgas analysis represented one experiment, although multiple runs were performed to determine best conditions for the ALR operation. Variations in the CER and OUR data were observed owing to variations associated with the *T. pubescens* culture used each time (e.g. dependent on the inoculum, etc) but similar trends were consistently observed with each run.

Table 6.4 Comparison of the maximum yields based on stoichiometric coefficients and experimental data for biomass formation on glucose by *T. pubescens*

Yield coefficient	Growth on glucose (theoretical)		Growth on glucose (experimental)
	Molar basis	Mass basis	Mass basis
$Y_{X/Glu}$	1.5	0.27 g.g^{-1}	0.27 g.g^{-1}
Y_{X/O_2}	5.9	5.89 g.g^{-1}	0.2 g.g^{-1}
$Y_{X/Peptone}$	1.9	1.98 g.g^{-1}	ND [#]
$Y_{CO_2/X}$	0.19	0.27 g.g^{-1}	2.81 g.g^{-1}

ND - not determined

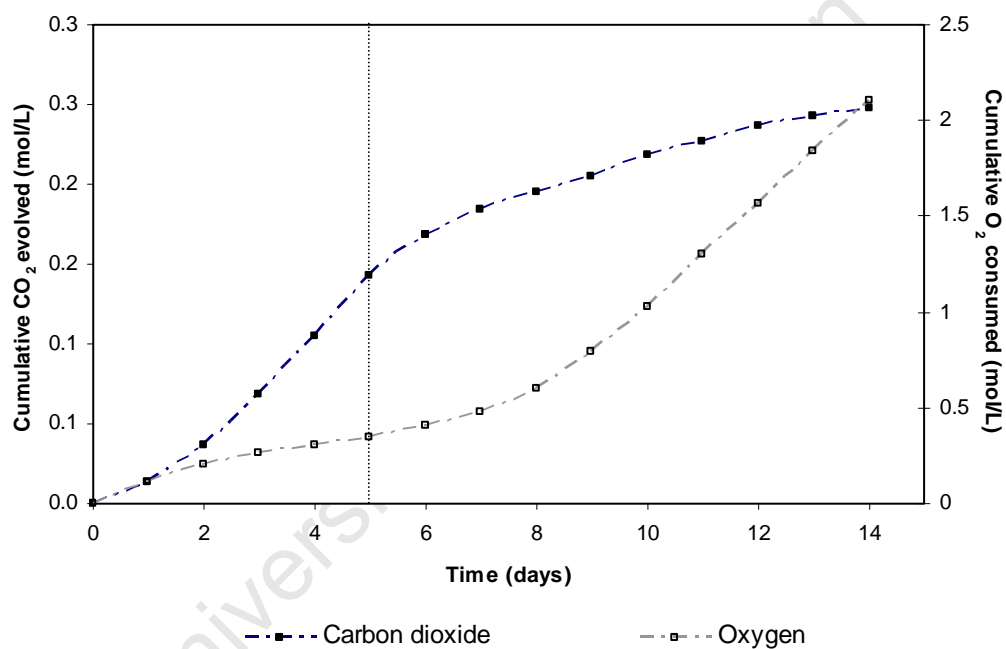
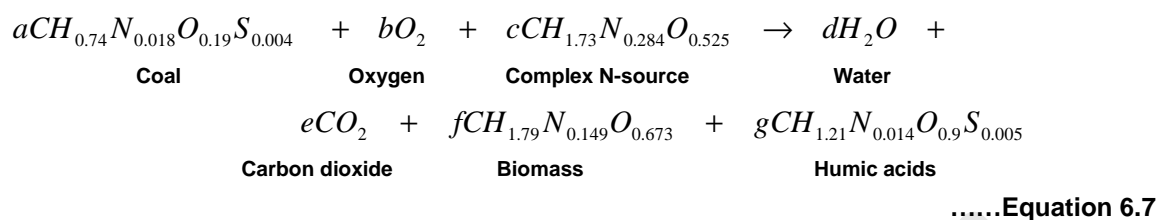


Figure 6.13 Measured cumulative carbon dioxide production and oxygen utilisation by *T. pubescens* grown on glucose and coal (added on day 5) in an ALR

6.3.4.2 Evaluation of *T. pubescens* growth on sub-bituminous coal in an airlift bioreactor

The next step in the kinetic analysis of coal bioconversion, by *T. pubescens* in the ALR, was to evaluate its growth on sub-bituminous coal. A stoichiometric analysis was performed as conducted for growth on glucose, in the previous section. The general form of the stoichiometric equation is given in below (Andrews, 1991):



The stoichiometry is dependent on the product spectrum, and certain assumptions were made in order to calculate the stoichiometric coefficients. Since only small changes were observed in the total organic carbon and total phenolic content, biomass, carbon dioxide and humic acids were assumed to be the major products formed from the bioconversion of coal as shown in Equation 6.7.

The formula for peptone (complex nitrogen source) was derived from the amino acid composition provided by the manufacturers, while formulae for coal and humic acid were calculated from the elemental composition of dried samples determined by microanalysis as described in Section 5.2.8 (Appendix C1 and C2).

The stoichiometric coefficients (*a-g*) were determined by using four element balances and the biomass yield ($Y_{X/S}$), calculated indirectly from the off- gas analysis data. The biomass yield on coal could not be measured experimentally due to the adhesion of fungal hyphae to coal particles, and biomass concentrations were calculated indirectly based on the oxygen utilisation rate (OUR) and the carbon dioxide evolution rate (CER) using the Pirt and Luedeking Piret equations as shown below. Equation 6.8 was integrated and solved using ODE45 Solver in Matlab software version 7.0.4. The biomass concentration was determined by inserting the experimentally determined OUR (or CER), the inverse of the growth yield coefficient and the yield coefficient for cell maintenance. Sample calculations are shown in Appendix C.5 and C.6.

$$\frac{-dO_2}{dt} = \left[\frac{1}{Y_{X/O_2}} * \frac{dX}{dt} \right] + mX$$

$$(-Y_{X/O_2}) \frac{-dO_2}{dt} = \frac{dX}{dt} + mX(Y_{X/O_2})$$

$$\frac{dX}{dt} = mX(Y_{X/O_2}) - (Y_{X/O_2}) \frac{dO_2}{dt} \quad \text{.....Equation 6.8}$$

Where $\frac{dO_2}{dt}$ = oxygen utilisation rate (OUR) (g O₂ .L⁻¹ day⁻¹), determined through experimental off-gas analysis.

$\frac{1}{Y_{X/O_2}}$ = the inverse of the growth yield coefficient (g O₂. g biomass⁻¹)

m = yield coefficient for cell maintenance (gO₂.g biomass⁻¹ day⁻¹)

$$m = \left(\frac{dO_2}{dt} \right) / X$$

X = biomass concentration (g/L)

Figure 6.14 shows the graphical representation of the predicted biomass yields, on glucose (day 0 to day 5) and coal (day 5 to day14) using OURs (during growth on glucose and later, coal) with different yield coefficients for cell maintenance (from duplicate runs in the airlift reactor), a theoretical OUR (Blanch and Clark, 1996) and CER data (during growth on glucose and, later coal). The experimental biomass yield on glucose (first 5 days) is also represented, indicating that the most accurate (and realistic) model is one in which the predicted biomass yield is based on the OUR and yield coefficient for cell maintenance of 0.55. The predicted maximum biomass yield was 5.13 g/L. The graph shows that a small change in the maintenance coefficient greatly affects the predicted biomass concentration. The maintenance coefficient, m , depends on the type of substrate consumed and the environmental conditions (Blanch and Clark, 1996). It should be noted that the gross assumption was made that the maintenance coefficient did not change even though the substrate changed from glucose (a simple sugar) to coal (a complex carbon source). Although inaccuracies in the OUR and CER data as well as in the mathematical model have been shown, a methodology of biomass prediction has been demonstrated. The accurate application of this

methodology still needs refinement, but the approach was valuable for the estimating the bioprocess yields of coal bioconversion by *T. pubescens*, in the present study.

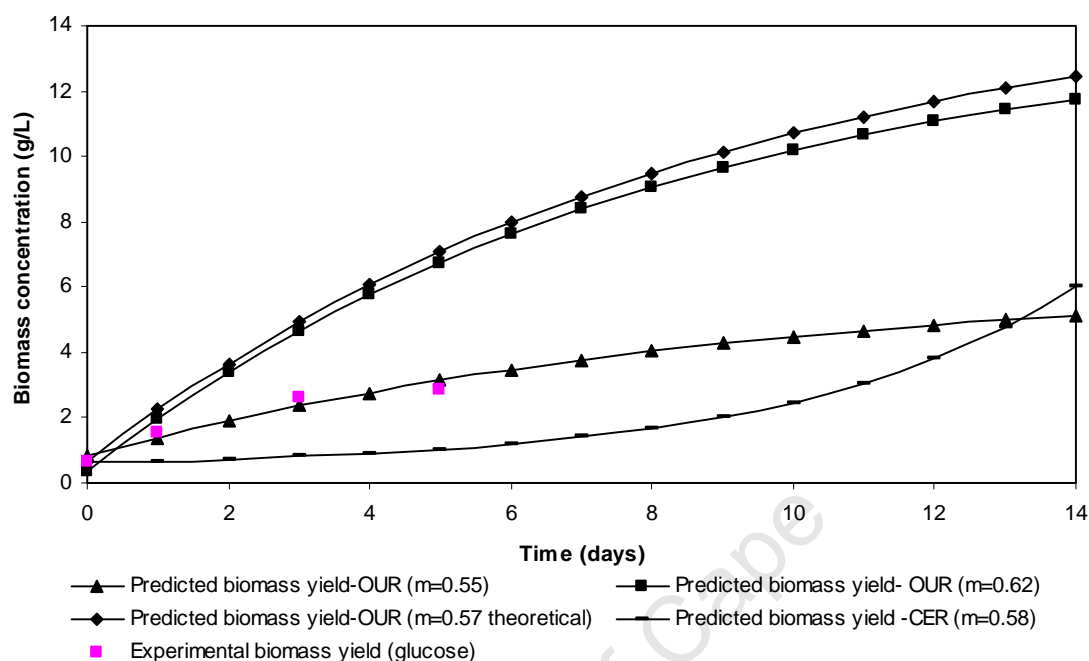


Figure 6.14 Biomass yield on coal (after 5th day) determined through a mathematical model based on oxygen utilisation rate (OUR), carbon dioxide evolution rate (CER) and experimental biomass yield on glucose (day 0-5) ('m' is the maintenance coefficient which was used in the calculation.)

The stoichiometric coefficients were determined from 4 element balances, experimental biomass yield based on OUR, experimental CO₂ yield based on CER and the experimental yield of humic acid (Table 6.2). The sulphur balance was disregarded due to the small concentrations of sulphur in coal and humic acid.

Elemental balances

$$\text{C: } a + c = e + f + g$$

$$\text{H: } 0.74a + 1.73c = 2d + 1.79f + 1.21g$$

$$\text{N: } 0.018a + 0.284c = 0.149f + 0.014g$$

$$\text{O: } 0.19a + 2b = 2e + d + 0.673f + 0.5g$$

$$\gamma: 4.33a - 4b + 3.83c = 4f + 3.398g$$

$f = 0.157$ - determined from experimental yield based on OUR and CER (using a maintenance coefficient of 0.62 obtained from the first run in the ALR)

$e = 0.37$ - determined from experimental CER

$g = 0.002$ - determined from experimental yield

By substituting f - g into the elemental balances,

$a = 0.475$

$c = 0.05$

$d = 0.08$

$b = 0.42$

Thus, Equation 6.9 was generated by using the predicted biomass yield based on OUR and the cell maintenance coefficient of 0.62, obtained from the first run in the ALR. Similarly, Equation 6.10 was generated by using a cell maintenance coefficient of 0.55, obtained from the second run in the ALR. (OUR and CER raw data, and sample calculations for the maintenance coefficients from different ALRs are shown in Appendix C.5 and C.6.) The theoretical yield coefficients based on the stoichiometric equation for biomass formation on sub-bituminous coal and glucose were compared and are shown in Table 6.5. The stoichiometric equations show that most of the coal carbon was used in the generation of biomass and carbon dioxide during its bioconversion by *T. pubescens* in the ALR. For an example, using Equation 6.9, 0.48 moles of coal was converted to 0.37 moles of carbon dioxide and 0.16 moles of biomass, with only 0.001 moles of humic acid produced. These results are in agreement with results on coal weight loss, observed in shake flask culture experiments, obtained from elemental analysis (Section 4.3.2.2.1) and results obtained from size exclusion chromatography (Section 6.3.3.3), and therefore confirmed that intermediate products from the bioconversion of coal by *T. pubescens* were mineralised, forming biomass and carbon dioxide.

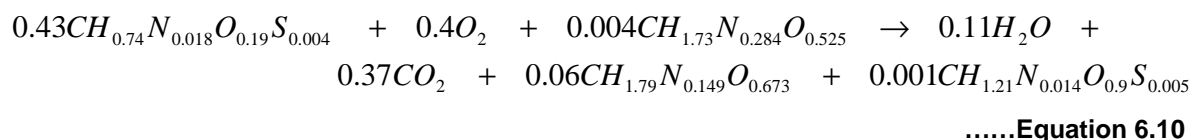
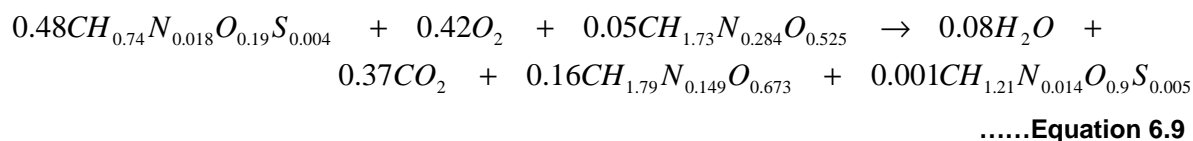


Table 6.5 Comparison of the maximum yields based on stoichiometric substrate consumption for biomass formation, on sub-bituminous coal and glucose, by *T. pubescens*.

Yield coefficient	<i>T. pubescens</i> growth on coal (Theoretical- Equation 6.9)		<i>T. pubescens</i> growth on coal (Theoretical- Equation 6.10)		<i>T. pubescens</i> growth on glucose (Theoretical)	
	Molar basis	Mass basis	Molar basis	Mass basis	Molar basis	Mass basis
$Y_{X/C-mole}$	0.33	0.52 g.g ⁻¹	0.14	0.22 g.g ⁻¹	1.5	0.27 g.g ⁻¹
Y_{X/O_2}	0.38	0.37 g.g ⁻¹	0.15	0.15 g.g ⁻¹	5.9	5.89 g.g ⁻¹
$Y_{X/Peptone}$	3.2	3.25 g.g ⁻¹	15	15.5 g.g ⁻¹	1.9	1.98 g.g ⁻¹
$Y_{CO_2/X}$	2.31	3.26 g.g ⁻¹	6.17	8.51 g.g ⁻¹	0.19	0.27g.g ⁻¹
$Y_{Humic\ acid/Coal}$	0.001	3.8 mg.g ⁻¹	0.001	3.2 mg.g ⁻¹	N/A	N/A

Gravimetric analysis of the biomass/coal sample obtained at the end of the run in the airlift reactor showed that 13.19 g L⁻¹ of residue, consisting of coal and fungal mycelia, remained. Subtracting the predicted maximum biomass yield of 5.13 g L⁻¹ from this value provided an estimate of the remaining coal in the reactor. 7.91 g L⁻¹ of the initial 10 g L⁻¹ coal remained, indicating a 20.9% bioconversion of sub-bituminous coal by *T. pubescens* in an airlift reactor. A comparison of the percentage coal bioconversion for the various reactor configurations and shake flask experiments is shown in Table 6.6. The best bioconversion of sub-bituminous coal was achieved in the airlift bioreactor, based on percentage coal bioconversion and production of laccase, possibly due to the increased mass transfer and higher enzyme activity in the airlift reactor. A higher percentage coal bioconversion was observed in the STRs at 2 and 10% coal loading, but the amount of time needed to achieve this was twice as long as what was required in the ALR.

Table 6.6 Percentage coal bioconversion by *T. pubescens* for various reactor configurations

Bioreactor configuration	Coal loading (% w/v)	Coal ave. particle size (μm)	Laccase activity (U mL^{-1})	pH	% Coal bioconversion	Method of analysis	Time (days)
STR	2	425	4.3	6.8	31	ave. particle size	18
	10	425	3.34	5 7.0	43	ave. particle size	18
FBR		425	1.17	nd	11	dry weight	6
PBR		2000	0.88	nd	4.9	dry weight	5
ALR	1	212-300	8.3	6.8	20.9	weight	9
Shake flask	1	~500	2.82	7.3	9.45	dry weight	9

nd- not determined

6.4 CONCLUSIONS

In this chapter, four different bioreactor configurations were investigated for the bioconversion of sub-bituminous coal by *T. pubescens*. These included a stirred tank slurry bioreactor, fluidised-and packed-bed bioreactors and an internal loop draught tube airlift bioreactor. Of the three fungal strains used in this project, *T. pubescens* was chosen for the bioreactor studies because it is a relatively new isolate, it is a faster grower, and exhibited superior laccase production in shake flask culture experiments. Although *T. versicolor* showed a slightly higher bioconversion of sub-bituminous coal in shake flask experiments than *T. pubescens*, the latter has not yet been investigated for the bioconversion of coal, and presented a novel system for investigation.

T. pubescens was shown to tolerate up to 10% (w/v) coal loading in the stirred tank slurry bioreactor while sustaining a relatively high laccase activity and percent bioconversion of coal, but the solids loading did not appear to have a key effect on the production of soluble, low molecular mass organic compounds (evidenced by a relatively unchanging total organic carbon and total phenolic content), which implied that regardless of the amount of substrate available, intermediate products was still taken up intracellularly and mineralised by the fungus.

The bioconversion of coal in the fluidised and packed bed bioreactors were better suited to a cell-free system, where the cell-free filtrate from a *T. pubescens* culture, cultivated separately from the bioreactors, was used for fluidisation and percolation, in the fluidised and packed-bed bioreactors, respectively. The presence of fungal mycelia in the system resulted in coal wash-out and fouling in the fluidised-bed and packed-bed bioreactors, respectively. A considerable percent coal bioconversion was obtained using the

cell-free system in only five days, and together with the results obtained from depolymerisation of coal by a cell-free filtrate from *T. pubescens* in shake flasks, this provides an incentive for the future development and optimisation of the system, with the aim of producing useful low molecular mass compounds from coal.

Apart from the superior performance of the airlift bioreactor in terms of coal bioconversion and laccase production by *T. pubescens*, the simplicity of the configuration and ease of operation allowed a wider range of analyses to be conducted. Measurement of the yield on humic acids and the molecular mass distribution of the products from the bioconversion of coal compared with those obtained during the cell-free treatment of coal in shake flask experiments, confirmed that the presence of fungal mycelia led to metabolism of products. This was further clarified by the higher production of CO₂, measured in the vent gas, when *T. pubescens* was grown on glucose as the primary carbon source, as compared to CO₂ produced when grown on coal.

Preliminary data on the kinetics of coal bioconversion by *T. pubescens* in an airlift reactor was also presented. The evaluation of growth of *T. pubescens* on glucose, and coal, was performed using a black-box approach to balance stoichiometric equations. A method for the indirect determination of biomass concentration in the presence of a solid substrate was developed and presented. This was based on a mathematical model using the Luedeking-Piret and Pirt equations for product formation and substrate consumption, respectively and the oxygen utilisation and carbon dioxide evolution rates. The sensitivity of the model to small changes in the yield coefficients for cell maintenance was demonstrated, and although inaccuracies in the O₂ and CO₂ concentration measurements were identified, the estimate of the biomass concentration allowed the calculation of the percentage coal bioconversion by *T. pubescens* in an airlift bioreactor. Furthermore, the theory that biomass and carbon dioxide were the major products of coal was bioconversion *T. pubescens*, was confirmed.

A comparison of the percentage coal bioconversion for the various reactor configurations and shake flask experiments was presented, showing that the best bioconversion of sub-bituminous coal was achieved in the airlift bioreactor. The combination of increased mass transfer (oxygen mass transfer, in particular), higher laccase activity, and smaller average particle size of coal in the airlift bioreactor resulted in the greater success of the bioconversion of coal by *T. pubescens*. In addition, an advantage of using the airlift design is the low energy cost associated with the absence of moving parts, while good mixing and aeration can still be achieved. Since different coal loadings and average particle size of coal were used in the bioreactor systems, a more rigorous comparison of these systems demands the design of an effective factorial experiment and is therefore recommended for future work.

CHAPTER 7: FINAL CONCLUSIONS AND RECOMMENDATIONS

This thesis described the first investigation into the bioconversion of a South African sub-bituminous coal by fungi. The research objectives have been met: microbial strains that had the potential to degrade low rank coal were acquired and the best strain capable of coal bioconversion was chosen; coal bioconversion products were identified and a better understanding of the bioconversion pathways was gained; and the most appropriate bioreactor configuration for the bioconversion of coal was chosen and used to provide a preliminary kinetic analysis of the yields of the coal bioconversion process. In meeting these research objectives, this project has contributed to the overall, long term goal of the broader research program, which is to produce valuable carbon intermediates from the bioconversion of low rank coal. The key findings of this study were that the bioconversion of SA sub-bituminous coal was best achieved by the white-rot fungus, *Trametes pubescens*, and that the laccase produced by this strain, in conjunction with veratryl alcohol acting a mediator, was able to depolymerise native sub-bituminous coal producing humic acids and other compounds of lower molecular mass. Further, coal carbon was mainly used by *T. pubescens* to produce biomass and carbon dioxide, and mineralization reactions were more prevalent when active fungal mycelia were present. Greater potential to produce valuable carbon intermediates from the bioconversion of sub-bituminous coal lies in use of the cell-free filtrate from *T. pubescens*.

Specifically, a number of environmental fungal isolates and culture strains were screened for their potential to degrade low rank coal and of these, the best three strains were chosen for further experiments. These included one of the indigenous environmental isolates, *Trichoderma atroviride* ES11, and two white rot fungal strains, *Trametes versicolor* and *Trametes pubescens*. The bioconversion of sub-bituminous coal was successfully demonstrated by each of these stains in liquid fermentation studies, and some of the degradation products were identified. Depolymerisation reactions were identified as the coal bioconversion mechanism of *T. pubescens*, and a better understanding of the biodegradation pathway was obtained by investigating bioconversion of coal by a cell-free enzyme preparation from this strain. In addition to this, an appropriate bioreactor configuration was identified for the bioconversion of sub-bituminous coal by *T. pubescens*.

The characterisation of South African sub-bituminous coal by elemental and proximate analyses showed that the coal was likely to be particularly resistant to biological attack, due to its low inherent moisture content, but the large amount of aromatic compounds present in the mobile phase of the coal could potentially be used to support microbial growth. For this reason, growth on coal as a sole carbon source could not be used as a sufficient indicator of the potential of microorganisms to degrade the macromolecular matrix of coal. Thus, a solid media screening system was developed to identify coal-degrading fungi that could produce extracellular ligninolytic enzymes, and that have the ability to cause depolymerisation, which is required for the biodegradation of large macromolecules. The ability of the

fungi to produce extracellular ligninolytic enzymes and/or depolymerise large macromolecules, such as those present in coal, was demonstrated by their ability to decolourise the polymeric anthraquinone-like dye, Poly R-478, and to cause bleaching of lignin, lignite and humic acids. A definite correlation between the two activities, *i.e.* production of extracellular ligninolytic enzymes and depolymerisation, was shown to exist, but both activities were limited to the basidiomycete fungi. *T. pubescens* showed superior degradative capabilities compared to the other fungi that were screened, and along with *T. versicolor*, also showed the ability to grow on coal macromolecules as a sole carbon source.

The environmental isolate, *T. atroviride* ES11 did not produce extracellular ligninolytic enzymes or depolymerise lignin or coal macromolecules in solid media, but it did have the ability to grow on particulate coal as sole carbon source. Thus, in order to gain clarity on how *T. atroviride* ES11 assimilates coal carbon for growth, further investigation, in liquid fermentations and resting cell reactions, were performed. Humic acids were released from the bioconversion of sub-bituminous coal by *T. atroviride* ES11, as well as small quantities of 4-hydroxyphenyl ethanol (tyrosol), 1,2-benzenediol (catechol) and 2-octenoic acid. However, the accumulation of low molecular mass compounds was not detected, and since actively respiring mycelia were required for coal bioconversion, the conclusion was drawn that these compounds were metabolised by *T. atroviride* ES11. In addition to this, coal bioconversion products were shown to adhere to fungal mycelia, possibly facilitating intracellular uptake and subsequent metabolism, leading to production of biomass. Neither the production of extracellular ligninolytic enzymes nor alkaline substances were found to be involved in the bioconversion of coal by this strain of *T. atroviride* (ES11); the mechanism by which it initially attacks coal is still, therefore, unknown and further research is required, possibly with regard to involvement of hydrolytic enzymes, such as esterases.

Further work with the *Trametes* spp. was continued because they possess extracellular ligninolytic enzymes, which enabled them to depolymerise lignin and coal macromolecules. Particular attention was given to investigations involving *T. pubescens*, being a relatively new isolate, and because the bioconversion of coal by this strain has not been reported. *T. versicolor* and *T. pubescens* were both able to degrade sub-bituminous coal in shake flask cultures, by producing extracellular laccase, in relatively large quantities. One of the major limitations in the coal bioconversion studies was the quantification of residual coal, due to attachment of coal particles to the fungal mycelia. A method was developed that involved the treatment of the combined coal and biomass residue, with nitric acid, to remove mycelia, followed by elemental analysis of the residual coal. Thus, 11.5 and 9.5 % coal carbon was shown to be degraded by *T. versicolor* and *T. pubescens*, respectively. This result was significant for a number of reasons. Firstly, most reports in literature only provide qualitative data on coal bioconversion, and secondly, most reports are on the bioconversion of lignite, which is more amenable to degradation than sub-bituminous coal and in most cases, the lignite used is oxidatively pre-treated to aid bioconversion by the microorganisms. The percentage coal bioconversion by the *Trametes* spp. compared favourably to values reported in literature for lignite bioconversion.

The bioconversion of lignite coal and asphaltene (representing sub-units of hard coals) was achieved by the *Trametes* spp. showing their versatility in their potential ability to degrade a range of coals. The potential for the removal or partial removal of asphaltene, a high molecular weight fraction found in oil, was shown, which would be economically attractive to the petroleum industry.

Humic acids were identified as being a product of coal bioconversion by *Trametes* spp., and an interesting relationship between laccase activity and the production of humic acids, was observed in shake flask culture experiments. The production of laccase was inhibited by the release of humic acids, and smaller amounts of humic acids were released when higher laccase activities were present, suggesting that mineralisation of humic acids occurred, leading to a lower recovery.

Next, the role of laccase in the bioconversion of coal was confirmed by using a cell-free filtrate with a high laccase activity, from *T. pubescens* shake flask cultures. Here, sub-bituminous coal was depolymerised to form humic acids, 1000 kDa to 530 kDa in size, and other products of lower molecular mass, less than 200 kDa. The involvement of non-enzymatic reactions in the bioconversion of coal was minor, resulting in the release of small amounts of humic acids in the control (containing autoclaved cell-free filtrate and coal), as compared with the amount produced when active laccase was present. Treatment of coal with the cell-free filtrate resulted in a 9.7 % reduction in coal carbon, while the low molecular mass compounds, produced from the depolymerisation of coal, were mineralized, as the reaction time continued. *T. pubescens* laccase has a high redox potential, suggesting that depolymerisation of coal, without an added redox mediator, could be achieved. However, veratryl alcohol, which was used as an inducer of laccase activity in the preparation of the cell-free extract from *T. pubescens*, was likely to have played a role, as a mediator in the depolymerisation reactions. Interestingly, *T. pubescens* appeared to produce veratryl alcohol from the complex growth medium used for cultivation, adding to the advantages of using this strain in the bioconversion of coal.

Lastly, the bioconversion of sub-bituminous coal by *T. pubescens* in an internal loop, draught-tube airlift bioreactor (ALR) was effectively demonstrated, achieving 21% coal bioconversion in 9 days. Among four reactor configurations that were tested, the airlift bioreactor proved to be the best suited for growth and laccase production by *T. pubescens* in a slurry system. Preliminary data on the kinetic yields of coal bioconversion by *T. pubescens* were also presented. Due to mycelial attachment to coal particles, an indirect method of biomass quantification, using oxygen utilization and carbon dioxide production rates was developed and applied. This, together with balanced stoichiometric equations, confirmed that sub-bituminous coal (and intermediate products), during its bioconversion by *T. pubescens*, is mainly used to produce biomass and carbon dioxide.

Although some technical difficulties were encountered while in operation of the fluidised-bed bioreactor, the data obtained from the system using the cell-free filtrate from *T. pubescens* to fluidise the bed was in

agreement with results from shake flasks, and showed considerable potential for the bioconversion of coal. Higher yields of humic acids were obtained in the absence of respiring fungal mycelia. Altogether, the bioreactor studies showed that a cell-free system is required for effective coal bioconversion resulting in the depolymerisation of coal, to yield intermediates of low molecular mass.

This project has shown that the bioconversion of sub-bituminous coal to yield valuable carbon intermediates is promising, and may prove to be valuable in the beneficiation of coal which is not used for energy, or coal fines which are wastes in the coal industry. Many avenues have been opened for future research. Because of the broad scope of this study, there remains the need for further detailed investigations. The following recommendations are made:

- **Microbial strain selection**

Use of white rot fungi and their ligninolytic systems in the bioconversion of coal has been shown to be the best route to obtain valuable low molecular mass intermediates. Genetic manipulation to block metabolic pathways and re-direct carbon flow may be required to improve product yields. However, genetic manipulation, to enhance product formation and accumulation, may be difficult in such eukaryotic systems. Thus, investigation of bacterial systems, which also produce oxidase enzymes, such as actinomycetes for an example, should be investigated. In addition, new microbial strains are discovered regularly, and screening for their coal bioconversion potential could deliver a strain with superior coal degrading capabilities.

- **Design of a continuous bioprocess (using *T. pubescens* to bioconvert coal, as an example)**

A continuous multi-step bioprocess system should be designed which possibly includes 1) an effective bioreactor, used to produce enzymes (laccase) and metabolites (veratryl alcohol) required for the bioconversion step, such as the ALR used in the present study, 2) a second bioreactor, used to contact the coal particles with the liquid containing the bioconversion agents (enzymes and mediators) in a continuous mode, and 3) continual extraction system for the removal of the desired products, such as a membrane extraction which allows the use of organic solvents, or adsorption. The recovery and separation of intermediate products from a complex mixture (expected from the bioconversion of coal) would be important for further biotransformation reactions.

- **Detailed bioprocess kinetic data**

A more stringent evaluation of the enzymatic coal bioconversion in terms of kinetic analysis is required, and would need to include substrate utilisation and inhibition constants, as well as yield coefficients and turnover numbers.

- **Biotransformation reactions**

Once carbon intermediates of lower molecular mass are recovered, they may be used in further biocatalytic reactions to yield specific value-added compounds, such as the conversion of phenols to catechols and dimers, which may have enhanced antioxidant properties. The production of natural antioxidants is a niche market, and thus the biotransformation of coal intermediates to yield antioxidants would add to the economic viability of the coal bioconversion process.

University of Cape Town

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APPENDIX A**Recipe for Trametes Defined Media (TDM) (adapted from Addleman & Archibald, 1993)**

Grams contained per 2 litres:

Glucose	20.0
Peptone	10.46
Potassium dihydrogen orthophosphate	4.0
Magnesium sulphate	1.0
Calcium chloride	0.2
Sodium chloride	0.58
2,2-Dimethyl succinate	2.6 mL
Veratryl alcohol	0.7 mL
Trace elements solution*	20 mL

*** Trace elements solution (100x concentration)**

Grams contained in 500mL dH₂O:

Iron sulphate	0.28
Copper sulphate	0.016
Zinc chloride	0.034
Manganese sulphate	0.169
Cobalt chloride	0.095
Nickel chloride	0.0012
Ammonium molybdate	0.309

APPENDIX B

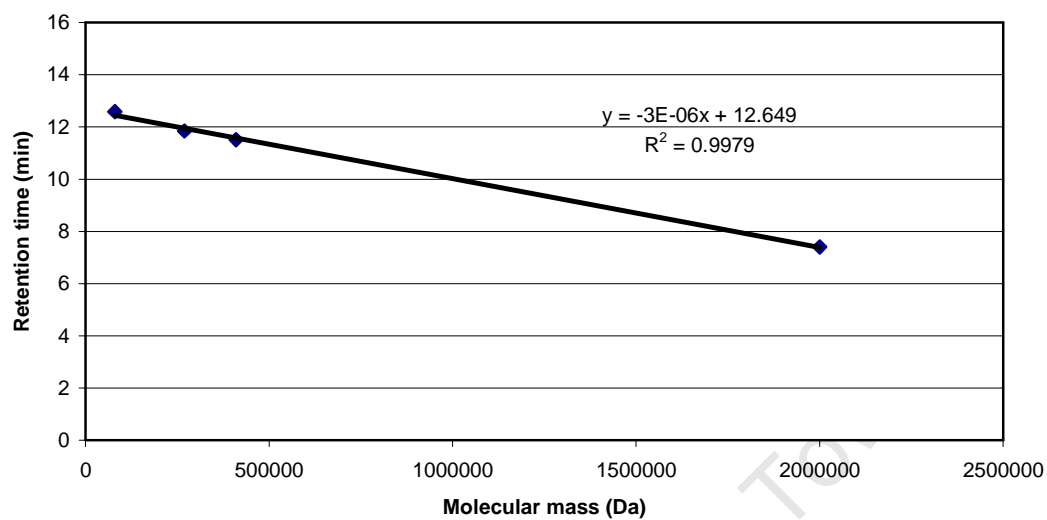


Figure B.1 Calibration curve of dextran molecular mass standards, $R^2 = 0.9979$

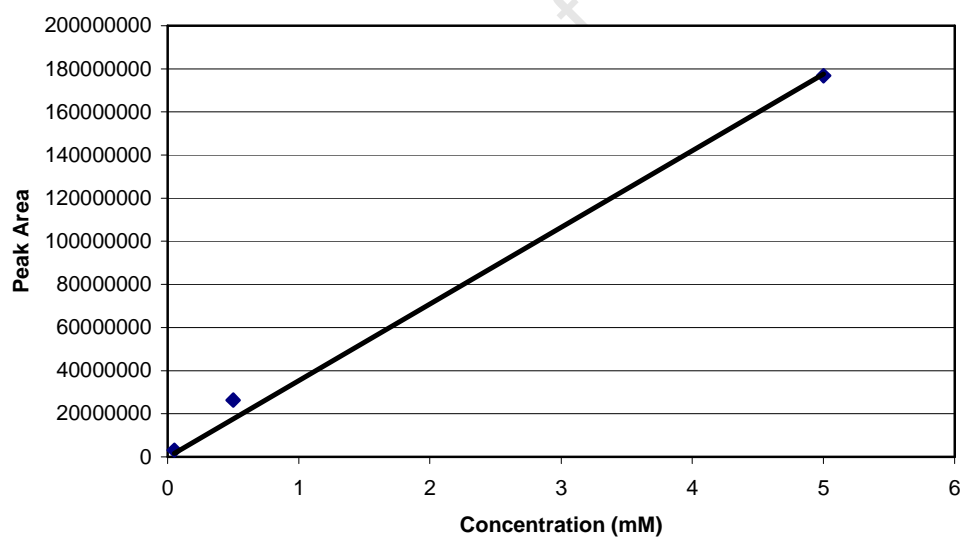


Figure B2. Veratryl alcohol standard curve, $R^2 = 0.9957$

APPENDIX C

C.1 Formulae derived from elemental composition

T. pubescens biomass formulaBiomass formula : $\text{CH}_{1.79}\text{N}_{0.149}\text{S}_{0.0062}\text{O}_{0.673}$

Elemental analysis of dried and pulverised biomass gave:

	Mass %	Basis 100g	Mol mass	Moles/100g	Normalise 1 mol C	Ash %
C	38.5	38.5	12	3.208	1.000	
H	5.75	5.75	1	5.750	1.792	
N	6.67	6.67	14	0.476	0.149	
S	0.64	0.64	32	0.020	0.00623	
O	31.99	31.99	16	1.999	0.623	
						16.54

Subbituminous coal formula

Coal formula: $\text{CH}_{0.74}\text{N}_{0.018}\text{S}_{0.004}\text{O}_{0.19}$

Elemental analysis of powdered coal gave:

	Mass %	Basis 100g	Mol mass	Moles/100g	Normalise 1 mol C	Ash %
C	59.13	59.13	12	4.93	1.00	
H	3.66	3.66	1	3.66	0.74	
N	1.24	1.24	14	0.09	0.018	
S	0.61	0.61	32	0.0191	0.004	
O	14.84	14.84	16	0.928	0.19	
						20.52

Humic acid formula

Humic acid formula: $\text{CH}_{1.21}\text{N}_{0.014}\text{S}_{0.005}\text{O}_{0.552}$

Elemental analysis of powdered humic acid gave:

	Mass %	Basis 100g	Mol mass	Moles/100g	Normalise 1 mol C	Ash %
C	42.86	42.86	12	3.57	1.000	
H	4.32	4.32	1	4.32	1.210	
N	0.68	0.68	14	0.05	0.014	
S	0.6	0.6	32	0.02	0.005	
O	31.51	31.51	16	1.97	0.552	
						20

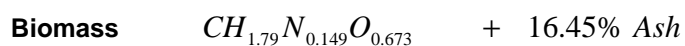
Peptone formula

Peptone formula: $\text{CH}_{1.73}\text{N}_{0.28}\text{S}_{0.000}\text{O}_{0.52}$

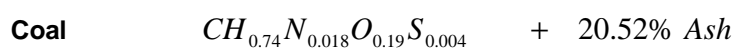
	Mass %	Basis 100g	Mol mass	Moles/100g	Normalise 1 mol C	Ash %
C			12	3.523	1.00	
H			1	6.0984	1.73	
N			14	0.9999	0.28	
S			32	0.0072	0.00	
O			16	1.8493	0.52	
						4.7

C.2 Molecular mass determinations

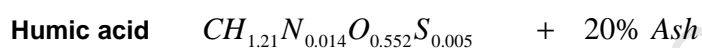
These formulae were derived from the elemental composition of each sample show in Appendix C1.



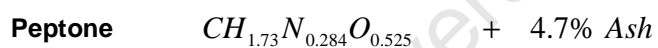
$$\begin{aligned} \text{MM}(CH_{1.79}N_{0.149}O_{0.673}) &= 1(12) + 1.79(1) + 0.149(14) + 0.673(16) \\ &= \frac{26.64}{0.8355} \\ &= 31.87 \text{ g / mol} \end{aligned}$$



$$\begin{aligned} \text{MM}(CH_{0.74}N_{0.018}O_{0.19}S_{0.004}) &= \frac{16.16}{0.7948} \\ &= 20.33 \text{ g / mol} \end{aligned}$$



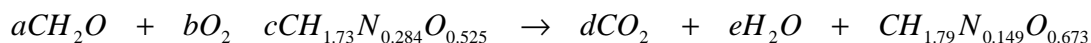
$$\begin{aligned} \text{MM}(CH_{1.21}N_{0.014}O_{0.552}S_{0.005}) &= \frac{22.40}{0.80} \\ &= 28 \text{ g / mol} \end{aligned}$$



$$\begin{aligned} \text{MM}(CH_{1.73}N_{0.284}O_{0.525}) &= \frac{26.12}{0.85} \\ &= 30.72 \text{ g / mol} \end{aligned}$$

C.3 Stoichiometric equations and elemental balance equations

GLUCOSE



$$\text{C: } a + c = d + 1$$

$$\text{H: } 2a + 1.73c = 2e + 1.79$$

$$\text{N: } 0.284c = 0.149$$

$$\text{O: } a + 2b + 0.525c = 2d + e + 0.673$$

$$\gamma: 4a - 4b + 3.83c = 4$$

5 unknowns, 5 equations.

$$a = d + 0.475$$

$$b = a - 0.95$$

$$c = 0.525$$

$$d = b + 0.022$$

$$e = a - 0.441$$

Using experimental biomass yield:

$$\frac{\Delta \text{ biomass}}{\Delta \text{ glucose}} = \frac{2.24g}{8.427g} = \frac{2.24g / 31.87g/mol}{8.427g / 180g/mol} = \frac{0.0703 \text{ mol}}{0.0468 \text{ mol}}$$

$$= 1.5 \text{ mol biomass per mol glucose or } 0.67 \text{ mol glucose gives 1 mol biomass}$$

So,

$$a = 0.667$$

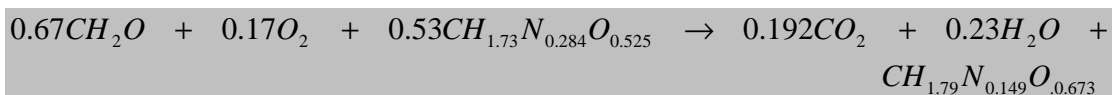
$$c = 0.525$$

Then

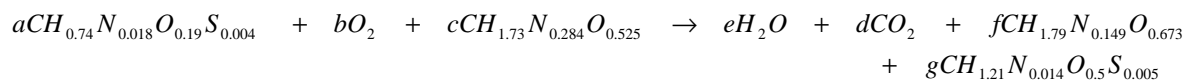
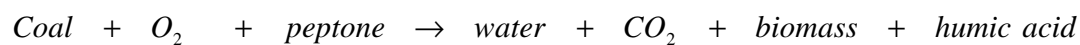
$$b = 0.169$$

$$d = 0.192$$

$$e = 0.226$$



COAL



$$\text{C: } a + c = d + f + g$$

$$\text{H: } 0.74a + 1.73c = 2e + 1.79f + 1.21g$$

$$\text{N: } 0.018a + 0.284c = 0.149f + 0.014g$$

$$\text{O: } 0.19a + 2b = 2d + e + 0.673f + 0.5g$$

$$\text{y: } 4.33a - 4b + 3.83c = 4f + 3.398g$$

5 unknowns, 5 equations

Disregard S-balance

$$f = 0.157 \text{ from experimental yield based on OUR and CER (m=0.62)}$$

$$d = 0.37 \text{ from experimental CER}$$

$$g = 0.002 \text{ from experimental yield}$$

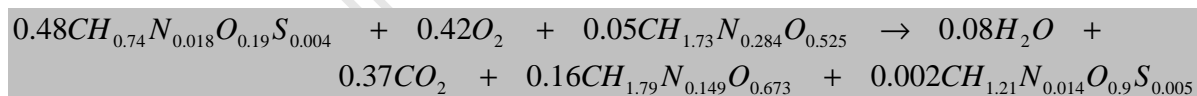
Then

$$a = 0.475$$

$$c = 0.05$$

$$e = 0.08$$

$$b = 0.42$$



And if

$$f = 0.06 \text{ from experimental yield based on OUR and CER (m=0.55)}$$

$$d = 0.37 \text{ from experimental CER}$$

$$g = 0.002 \text{ from experimental yield}$$

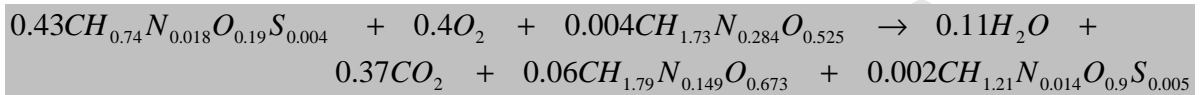
Then

$$a = 0.428$$

$$c = 0.004$$

$$e = 0.107$$

$$b = 0.404$$



C.4 Theoretical yields based on stoichiometric equations

Carbon source = GLUCOSE

$$Y_{X/Glucose} = 1/1 = \frac{1 \text{ mol biomass}}{0.667 \text{ mol glucose}} = \frac{31.87}{120} = 0.265 \text{ g biomass / g glucose}$$

$$Y_{X/O_2} = \frac{1(31.87)}{0.169(32)} = \frac{31.87}{5.405} = 5.89 \text{ g biomass / g } O_2$$

$$Y_{X/Peptone} = \frac{1(31.87)}{0.525(30.72)} = \frac{31.87}{16.128} = 1.98 \text{ g biomass / g peptone}$$

$$Y_{X/CO_2} = \frac{1(31.87)}{0.192(44)} = \frac{31.87}{8.448} = 3.77 \text{ g biomass / g } CO_2$$

Carbon source = COAL (m=0.62)

$$Y_{X/Coal} = \frac{0.157 (31.87)}{0.475 (20.33)} = \frac{5}{9.66} = 0.518g \text{ biomass} / g \text{ coal}$$

$$Y_{X/O_2} = \frac{0.157(31.87)}{0.42 (32)} = \frac{5}{13.44} = 0.372g \text{ biomass} / g O_2$$

$$Y_{X/Peptone} = \frac{0.157 (31.87)}{0.05 (30.72)} = \frac{5}{1.536} = 3.25g \text{ biomass} / g \text{ peptone}$$

$$Y_{X/CO_2} = \frac{0.157(31.87)}{0.37 (44)} = \frac{5}{16.28} = 0.307g \text{ biomass} / g CO_2$$

$$Y_{Humic\ acid / Coal} = \frac{0.002(28)}{0.475 (20.33)} = \frac{0.056}{9.66} = 6mg \text{ humic acid} / g \text{ coal}$$

Carbon source = COAL (m=0.55)

$$Y_{X/Coal} = \frac{0.06 (31.87)}{0.43 (20.33)} = \frac{1.91}{8.74} = 0.22g \text{ biomass} / g \text{ coal}$$

$$Y_{X/O_2} = \frac{0.06 (31.87)}{0.4 (32)} = \frac{1.91}{12.8} = 0.15g \text{ biomass} / g O_2$$

$$Y_{X/Peptone} = \frac{0.06 (31.87)}{0.004 (30.72)} = \frac{1.91}{0.123} = 15.53g \text{ biomass} / g \text{ peptone}$$

$$Y_{CO_2/X} = \frac{0.37(44)}{0.06 (31.87)} = \frac{16.28}{1.91} = 8.52g \text{ biomass} / g CO_2$$

$$Y_{Humic\ acid / Coal} = \frac{0.001(28)}{0.43 (20.33)} = \frac{0.028}{8.74} = 3.2mg \text{ humic acid} / g \text{ coal}$$

C.5 Oxygen utilisation rate (OUR) and carbon dioxide evolution rate (CER) calculations

$$OUR = \frac{\text{rate of } O_2 \text{ consumption}}{\text{reactor volume}} \left(\frac{\text{mol}}{\text{min} \cdot L} \right)$$

$$r_{O_2, \text{Consumed}} = r_{O_2, \text{in}} - r_{O_2, \text{out}} \left(\frac{\text{mol}}{\text{min}} \right)$$

$$\begin{aligned} r_{O_2, \text{in}} &= \% O_{2, \text{in}} * \text{total moles of air}_{\text{in}} \\ &= \% O_{2, \text{in}} * \frac{P \cdot Q_{\text{air, in}}}{R \cdot T} \end{aligned}$$

$$r_{O_2, \text{out}} = \% O_{2, \text{out}} * \underbrace{\text{total moles of air}_{\text{out}}}_{r_{\text{air, out}}} \dots\dots\dots 1$$

Recognise that N₂ is not consumed i.e. $r_{N_2, \text{in}} = r_{N_2, \text{out}}$

$$\begin{aligned} r_{N_2, \text{in}} &= \% N_{2, \text{in}} * r_{\text{air, in}} \\ &= \% N_{2, \text{in}} * \frac{P \cdot Q_{\text{in}}}{R \cdot T} \\ &= (100 - \% O_{2, \text{in}}) * \frac{P \cdot Q_{\text{in}}}{R \cdot T} \end{aligned}$$

$$r_{N_2, \text{out}} = \% N_{2, \text{out}} * r_{\text{air, out}}$$

$$(100 - \% O_{2, \text{in}}) * \frac{P \cdot Q_{\text{in}}}{R \cdot T} = (100 - \% O_{2, \text{out}}) * r_{\text{air, out}}$$

$$r_{\text{air, out}} = \left(\frac{100 - \% O_{2, \text{in}} - \% CO_{2, \text{in}}}{100 - \% O_{2, \text{out}} - \% CO_{2, \text{out}}} \right) * \frac{P \cdot Q_{\text{in}}}{R \cdot T}$$

$$r_{O_2, \text{out}} = \% O_{2, \text{out}} * r_{\text{air, out}} \dots\dots\dots 2$$

$$r_{O_2, \text{out}} = \% O_{2, \text{out}} * \left(\frac{100 - \% O_{2, \text{in}} - \% CO_{2, \text{in}}}{100 - \% O_{2, \text{out}} - \% CO_{2, \text{out}}} \right) * \frac{P \cdot Q_{\text{in}}}{R \cdot T}$$

$$OUR = \frac{r_{O_2, \text{in}} - r_{O_2, \text{out}}}{V_m}$$

$$= \frac{\% O_{2, in} - (\% O_{2, out} * \left(\frac{100 - \% O_{2, in} - \% CO_{2, in}}{100 - \% O_{2, out} - \% CO_{2, out}} \right))}{V_m \cdot 100} * \frac{P \cdot Q_{in}}{R \cdot T}$$

$$\begin{aligned} \frac{P \cdot Q_{in}}{R \cdot T} &= \frac{1.9738 \text{ atm} * 3 \text{ L} \cdot \text{min}^{-1}}{0.082057 \text{ L} \cdot \text{atm} \cdot \text{K}^{-1} \cdot \text{mol}^{-1} * 298 \text{ K}} \\ &= 0.242 \text{ mol} \cdot \text{min}^{-1} \end{aligned}$$

$$OUR = (\text{mol} \cdot \text{min}^{-1} \text{ L}^{-1})$$

Carbon dioxide formation calculations

$$CO_2 \text{ formed} = (Q_{out} * C_{CO_2, out}) - (Q_{in} * C_{CO_2, in})$$

$$CO_{2, in} \text{ and } CO_{2, out} = \text{ppm } CO_2 * \frac{1}{1^{-06} * 44} * 1.98 \quad \rho_{CO_2} = 1.98 \text{ g / L}$$

$$\begin{aligned} Q_{out} &= r_{air, out} * 12.39 \text{ L} \cdot \text{mol}^{-1} \text{ (Pressure of inlet air 1.9738 atm)} \\ &= (\text{L} \cdot \text{min}^{-1}) \end{aligned}$$

C.6 Indirect biomass determination using Pirt and Luedeking-piret equation

C.6.1 Using OUR

$$\frac{-dO_2}{dt} = \left[\frac{1}{Y_{X/O_2}} * \frac{dX}{dt} \right] + mX$$

$$\left(-Y_{X/O_2} \right) \frac{-dO_2}{dt} = \frac{dX}{dt} + mX(Y_{X/O_2})$$

$$\frac{dX}{dt} = mX(Y_{X/O_2}) - (Y_{X/O_2}) \frac{dO_2}{dt} \quad \dots \dots \text{Equation C.6.1}$$

Where $\frac{dO_2}{dt}$ = oxygen utilisation rate (OUR) (g O₂ .L⁻¹ day⁻¹), determined through experimental off-gas analysis.

$\frac{1}{Y_{X/O_2}}$ = the inverse of the growth yield coefficient (g O₂. g biomass⁻¹)

m = yield coefficient for cell maintenance (gO₂.g biomass⁻¹ day⁻¹) $m = \left(\frac{dO_2}{dt} \right) / X$

X = biomass concentration (g/L)

C.6.2 Using CER

$$\frac{dCO_2}{dt} = \left[\frac{1}{Y_{X/CO_2}} * \frac{dX}{dt} \right] + mX$$

$$(Y_{X/CO_2}) \frac{dCO_2}{dt} = \frac{dX}{dt} + mX(Y_{X/CO_2})$$

$$\frac{dX}{dt} = (Y_{X/CO_2}) \frac{dCO_2}{dt} - mX(Y_{X/CO_2}) \quad \dots \dots \text{Equation C.6.2}$$

Where $\frac{dCO_2}{dt}$ = carbon dioxide evolution utilisation rate (CER) (g CO₂.L⁻¹ day⁻¹), determined through experimental off- gas analysis.

$\frac{1}{Y_{X/CO_2}}$ = the inverse of the growth yield coefficient (g CO₂. g biomass⁻¹)

m = yield coefficient for cell maintenance (gCO₂.g biomass⁻¹ day⁻¹) $m = \left(\frac{dCO_2}{dt} \right) / X$

X = biomass concentration (g/L)

Equation C.6.1 and C.6.2 were integrated and solved using ODE45 Solver in Matlab software version 7.0.4. The biomass concentration was determined by inserting the experimentally determined oxygen utilisation rate, the inverse of the growth yield coefficient and the yield coefficient for cell maintenance.

C.6.3 Code from Matlab model used to calculate biomass concentration using OUR data from ALR (maintenance coefficient = 0.62)

```

close all; clear all; clc;

t = linspace(0, 14, 15);
%t = [1 2 3 4 5 6 7 8 9 10 11 12 13 14]; % [h]

X0 = .37;

%OUR = [0.00011687 7.80419E-05 6.50839E-05 4.07731E-05 2.84206E-05 2.56468E-05
4.12775E-05 5.39211E-05 8.66545E-05...
0.000127277 0.000168 0.000189717 0.000181089 0.000191072 0.00018626];

%X = exp((-B/A)*t).*trapz(t, (B/(A^2))*OUR.*exp((B/A)*t)) + X0*exp((-B/A)*t);

[T X] = ODE45(@ODEFN,t,X0);
plot(T, X), title('Biomass concentration'),xlabel('Time
[Days]'),ylabel('Biomass Concentration[g/L]');

function dX = ODEFN(t,X)

OUR = [0.00011687 7.80419E-05 6.50839E-05 4.07731E-05 2.84206E-05 2.56468E-05
4.12775E-05 5.39211E-05 8.66545E-05...
0.000127277 0.000168 0.000189717 0.000181089 0.000191072 0.00018626];
A = 5; % 1/Yox
B = 0.62%240/80; %maintenance

for i = 1:14
    if t == i
        OURi = -1*OUR(i+1);
    else if t < i
        OURi = -1*OUR(i);
    end
end
end

dX = -X*(B/A) - 32*1440*OURi/A;

```

C.6.4 Code from Matlab model used to calculate biomass concentration using OUR data from ALR (maintenance coefficient $m=0.55$)

```
close all; clear all; clc;

t = linspace(0, 14, 15);
%t = [1 2 3 4 5 6 7 8 9 10 11 12 13 14]; % [h]

X0 = .81;

%OUR = [0.00011687 7.80419E-05 6.50839E-05 4.07731E-05 2.84206E-05 2.56468E-05
4.12775E-05 5.39211E-05 8.66545E-05...
%0.000127277 0.000168 0.000189717 0.000181089 0.000191072 0.00018626];

%X = exp((-B/A)*t).*trapz(t, (B/(A^2))*OUR.*exp((B/A)*t)) + X0*exp((-B/A)*t);

[T X] = ODE45(@ODEFN,t,X0);
plot(T, X), title('Biomass concentration'),xlabel('Time
[Days]'),ylabel('Biomass Concentration[g/L]');

function dX = ODEFN(t,X)

OUR = [7.5222E-05 5.39463E-05 4.1535E-05 1.77506E-05 -2.02063E-05 -1.47991E-05
-8.23396E-05 -5.2355E-05...
3.33994E-05 5.27897E-05 -8.58066E-06 3.9776E-05 6.99684E-05 7.5396E-05
6.98409E-05 6.94908E-05];
A = 5; % 1/Yox
B = 0.55%240/80; %maintenance

for i = 1:14
    if t == i
        OURi = -1*OUR(i+1);
    else if t < i
        OURi = -1*OUR(i);
    end
end
end
```

C.6.5 Code from Matlab model used to calculate biomass concentration using CER data from ALR (maintenance coefficient = 0.56)

```
close all; clear all; clc;

t = linspace(0, 14, 15);
%t = [1 2 3 4 5 6 7 8 9 10 11 12 13 14]; % [h]
```

```

X0 = .64;

%OUR = [0.00011687 7.80419E-05 6.50839E-05 4.07731E-05 2.84206E-05 2.56468E-05
4.12775E-05 5.39211E-05 8.66545E-05...
%0.000127277 0.000168 0.000189717 0.000181089 0.000191072 0.00018626];

%X = exp((-B/A)*t).*trapz(t, (B/(A^2))*OUR.*exp((B/A)*t)) + X0*exp((-B/A)*t);

[T X] = ODE45(@ODEFN,t,X0);
plot(T, X), title('Biomass concentration'),xlabel('Time
[Days]'),ylabel('Biomass Concentration[g/L]');

function dx = ODEFN(t,X)

OUR = [0.016396277 0.048511505 0.08129949 0.107339805 0.130071507 0.133044502
0.08784337 0.055413841 0.039297488 ...
0.033561534 0.04658913 0.032681349 0.032499757 0.021754345 0.017472348]; % CER
A = 2.18; % 1/Yox
B = 0.56 %240/80; %maintenance

for i = 1:14
    if t == i
        OURi = -1*OUR(i+1);
    else if t < i
        OURi = -1*OUR(i);
    end
end
end

dx = -(-X*(B/A) - (44*OURi/3.5)/A);

```

Table C.6.1 Raw data and calculated CER from off-gas analysis during the bioconversion of coal by *T. pubescens* in an ALR (run #1)

Time (hours)	Time (Days)	CO ₂ (ppm)	CO ₂ OUT CO ₂ mol/L	CO ₂ in CO ₂ mol/L	O ₂ :N ₂ vol %	F _{out} (mol/min)	F _{out} (L/min)	CO ₂ form rate (mol/min)	Amt CO ₂ (mol/day)	Cumm. CO ₂ formed (mol)	Cumm. CO ₂ formed (g)
0	0	482	0.00002169	0.000017865	20.59	0.241800704	2.99591	0.0000114	0.016396277	0.00	0.00
24	1	646	0.00002907		20.6	0.242334942	3.00253	0.0000337	0.048511505	0.05	2.13
48	2	813	0.000036585		20.58	0.242788123	3.00814	0.0000565	0.08129949	0.13	5.71
72	3	945	0.000042525		20.58	0.243196508	3.01320	0.0000745	0.107339805	0.24	10.43
96	4	1060	0.0000477		20.57	0.243522343	3.01724	0.0000903	0.130071507	0.37	16.16
120	5	1075	0.000048375		20.57	0.243568962	3.01782	0.0000924	0.133044502	0.5003	22.01
144	6	846	0.00003807		20.6	0.242951931	3.01017	0.0000610	0.08784337	0.59	25.88
168	7	681	0.000030645		20.62	0.242504301	3.00463	0.0000385	0.055413841	0.64	28.32
192	8	599	0.000026955		20.6	0.242190404	3.00074	0.0000273	0.039297488	0.68	30.04
216	9	570	0.00002565		20.56	0.241978522	2.99811	0.0000233	0.033561534	0.72	31.52
240	10	637	0.000028665		20.5	0.242	2.99838	0.0000324	0.04658913	0.76	33.57
264	11	566	0.00002547		20.49	0.241751697	2.99530	0.0000227	0.032681349	0.80	35.01
288	12	565	0.000025425		20.5	0.241779261	2.99565	0.0000226	0.032499757	0.83	36.44
312	13	510	0.00002295		20.5	0.241610913	2.99356	0.0000151	0.021754345	0.85	37.40
336	14	488	0.00002196		20.51	0.241574213	2.99310	0.0000121	0.017472348	0.87	38.16
Blank		397			20.74						

Table C.6.2 Raw data and calculated OUR from off-gas analysis during the bioconversion of coal by *T. pubescens* in an ALR

Day	O ₂ :N ₂ vol %	100-%O _{out} -%CO ₂ out	OUR (mol/L.min)	O ₂ cons (mol/L.day)	Cumulative O ₂ (mol/L)	Cumulative O ₂ (mol)	Cumulative O ₂ (g)
0	20.59	78.928	0.166956593	0.00011687	0	0	0
1	20.6	78.754	0.111488432	7.80419E-05	0.11238034	0.393331189	12.58659806
2	20.58	78.607	0.092976961	6.50839E-05	0.206101117	0.721353909	23.08332508
3	20.58	78.475	0.05824734	4.07731E-05	0.264814435	0.926850524	29.65921677
4	20.57	78.37	0.040600868	2.84206E-05	0.30574011	1.070090385	34.24289233
5	20.57	78.355	0.036638249	2.56468E-05	0.342671465	1.199350128	38.37920409
6	20.6	78.554	0.058967844	4.12775E-05	0.402111052	1.407388681	45.03643778
7	20.62	78.699	0.077030204	5.39211E-05	0.479757497	1.679151239	53.73283965
8	20.6	78.801	0.123792084	8.66545E-05	0.604539917	2.115889711	67.70847075
9	20.56	78.87	0.181824775	0.000127277	0.787819291	2.757367517	88.23576055
10	20.5	78.863	0.24	0.000168	1.029739291	3.604087517	115.3308005
11	20.49	78.944	0.271023637	0.000189717	1.302931117	4.560258908	145.9282851
12	20.5	78.935	0.258698929	0.000181089	1.563699638	5.472948732	175.1343594
13	20.5	78.99	0.272959868	0.000191072	1.838843185	6.435951147	205.9504367
14	20.51	79.002	0.266086302	0.00018626	2.107058177	7.374703619	235.9905158

APPENDIX D

The following is a copy of the final revised manuscript that was agreed on by the authors prior to re-submission to Fuel Processing Technology journal. The journal printed the old version of the manuscript in error, which included the omission of an author (Oboirien, B.O., Burton, S.G., Cowan, D.A and Harrison, S.T.L. (2008) The effect of the particulate phase on coal biosolubilisation mediated by *Trichoderma atroviride* in a slurry bioreactor. *Fuel Proc Technol* **89**:123-130). Subsequently, the corresponding author, S.T.L. Harrison, requested that an erratum be published in notification of this mistake. As yet the editor of the journal has not responded to the request.

University of Cape Town

The Effect of the Particulate Phase on Coal Biosolubilisation mediated by *Trichoderma atroviride* in a Slurry Bioreactor

B.O. Oboirien, C.J. Vengadajellum, S.G. Burton, D.A. Cowan[#] and S.T.L. Harrison*

Bioprocess Engineering Research Unit, Department of Chemical Engineering,

University of Cape Town, Rondebosch, 7701, South Africa

[#] Department of Biotechnology, University of the Western Cape, Bellville, South Africa

Abstract

Low rank coal is currently under-utilised because of its low calorific value and high moisture and sulphur content. Its solubilisation by both bacterial and fungal cultures has been reported, the latter more commonly. Coal biosolubilisation processes have potential to convert low rank coal to either a clean, cost-effective energy source or complex aromatic compounds for biocatalytic conversion to value-added products. This can lead to an increased utilisation of low rank coal. In this study, the key variables of the slurry that affect biosolubilisation of low rank coal by *Trichoderma atroviride* in submerged culture were investigated. Results showed that the key operating variables that influence coal biosolubilisation in the slurry bioreactor are coal loading and particle size, factors affecting available surface area. The optimum coal loading occurred between 5 and 10 % (w/v); an increase above this optimum led to inhibition of the fungal culture of *Trichoderma atroviride* (ES11) by fragmentation of the fungal mycelium. A decrease in the particle size fraction led to an increase in the degree of coal solubilisation. Coal biosolubilisation was shown to increase 4-fold when particle size was decreased from 600-850 μm to 150-300 μm . A 28 % biosolubilisation of coal of 150-300 μm , characterised by a surface specific area of 2.17 $\text{cm}^2 \text{g}^{-1}$, was measured as coal weight loss over 14 days at solids loading at 5%. This can be compared with a 7.8% coal weight loss at 600-850 μm diameter (0.54 $\text{cm}^2 \text{g}^{-1}$). Soluble phenolic compounds are not a significant product of the coal biosolubilisation process. The change in pH observed in the presence of both coal and fungi was not directly correlated to the extent of coal solubilisation. While soluble intermediates were observed as total organic carbon, further metabolism resulted in complete oxidation of a significant fraction of the coal to CO_2 .

Key words: *Trichoderma atroviride* (ES 11), Low rank coal, particle size fraction, coal loading, coal biosolubilisation

* The author to whom all correspondence should be sent

Introduction

Fossil fuels remain the primary source of energy, with renewable energy sources providing an increasing, but small, contribution to the world's energy needs. Coal represents one of the world's most abundant fossil energy resources. The world's coal reserves are estimated at 1.53×10^{20} Btu or 71.4% of the world fossil fuel resource (1). Thermal and chemical processes currently dominate conversion of coal to liquid and gaseous energy products. These generally require extreme conditions of temperature, pressure and chemical environment, hence efficient processing is limited to coals of high calorific value (2). Coal is used for electricity generation, generation of industrial steam, transportation, residential heating, and conversion to liquid fuels. Coke (fixed carbon residue) from carbonisation of coal is used in metallurgical fabrication processes.

Low rank coal is not used significantly in the above-mentioned processes through current coal conversion technologies owing to its low calorific value and high moisture and sulphur content. However, the reserves of low rank coal are substantial (2). World reserves of low rank coal in 2002 were 47 % of the total recoverable reserves of coal, while total low rank coal production in 2001 was approximately 28% of the total production of coal worldwide (3). Microbial solubilisation of low rank coal at ambient temperature and pressure has been demonstrated (4-6) and reported to yield useful oxidised products (7). These products have potential use as substrates in biotransformation processes for the production of value added compounds such as antioxidants (7). The remaining unsolubilised coal fractions have been postulated to be enriched in aliphatic compounds with a low water and ash content (8). The residual unsolubilised coal is characterised by a higher heating value. This can then be used as a clean and cost effective energy source (8).

Coal biosolubilisation has been determined qualitatively by the colouration of the supernatant measured by absorbance at 450 nm (3, 9-12). The liquid product from coal biosolubilisation includes humic and fulvic acids which impart a dark-brown colour and display a maximum absorbance at 450 nm.

The biosolubilisation of coal necessitates use of either a packed bed or slurry process to provide contact between the microbial phase and its enzyme products and coal. The particle size fraction influences performance of both reactor systems while coal loading is expected to impact the slurry process.

The literature presents a contradiction on the effect of particle size on coal biosolubilisation. Cohen *et al.* (9) observed that a decrease in particle size across the range 250 to 105 μm resulted

in a higher degree of biosolubilisation when pre-treated coal was incubated in cell-free culture. This is in agreement with increased surface area and surface-based reaction. Conversely, Gokacy *et al.* (12) observed that an increase in particle size across the range 150 to 2000 μm led to a higher degree of biosolubilisation. Earlier research on coal biosolubilisation was carried out across the range of 0.1 to 1% (w/v) coal loading (6, 9). Gokacy *et al.* (12) investigated the effect of increased coal loading on coal biosolubilisation. They observed that 10 % (w/v) coal loading resulted in a higher release of colour compounds into the supernatant than 5 % (w/v) coal loading.

Quigley *et al.* (13) reported on the correlation between pH and biosolubilisation of oxidised, low rank coal. They observed an increase in pH from 6.5 to 7.5 and corresponding increase in absorbance at 450 nm from 0.0 to 0.5 during solubilisation of treated coal by *Streptomyces setonii*. Holker *et al.* (14) also suggested that the growth of fungi on carboxylic groups from the coal macromolecule leads to an increase in culture pH. Conversely, Larboda *et al.* (10, 15) reported that biosolubilisation of untreated coal does not influence pH. They observed that variation in pH of the supernatant did not correspond to the observed increase in absorbance.

In this study, we report on the biosolubilisation of low rank coal sourced from SASOL (SA) by the fungus *Trichoderma atroviride* in a slurry system at shake flask scale. The study focuses on quantifying dominant soluble products, as well as the effect of solids loading and particle size on microbial solubilisation performance with the view to accessing potential uses as a process.

Materials and Methods

Coal

Sub-bituminous coal from SASOL (SA) was used. Coal samples were dry sieved using laboratory sieves to prepare specific size fractions. Through this, the removal of fines that are often present in the broad, naturally occurring size fractions was ensured. Nominal size fractions of 1500-2000 μm , 600-850 μm and 150-300 μm were prepared. The dry coal samples were autoclaved at 120°C for 20 min. No further pretreatment was performed.

Micro-organism

The fungal strain *Trichoderma atroviride* ES 11, isolated from soil on 1% particulate coal as sole carbon source (16), was obtained from the laboratory of Prof. D Cowan at the University of the Western Cape, South Africa. It was maintained on agar slopes (3% agar) on the standard growth media (defined below) at 4°C for up to 6 months.

Inoculum preparation

The pre-inoculum was prepared by culturing the fungus from the stock culture on agar plates using the growth medium. Following 5 days of culture, five plugs of fungal culture were cut from the stock plates with a sterile Pasteur pipette and inoculated into 100 ml sterile growth media containing 0.1% coal. Ten glass beads (6mm in diameter) were added and the culture was grown for 4 days at 28°C in an orbital incubator shaker at 120 rpm. The inoculum was prepared by transferring a 10 ml aliquot of the pre-inoculum culture into 100 ml of fresh growth medium and cultured for 2 days at 28°C with agitation at 120 rpm.

Growth medium and culture conditions

The growth medium used for stock culture, inoculum development and experiment contained (per litre): 1 g $\text{NH}_4(\text{SO}_4)$, 3 g malt extract, 0.52 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 7.6 g KH_2PO_4 , 0.005 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.003 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. The standard growth medium was supplemented with 10 g glucose per litre, unless otherwise stated.

Where coal was used as carbon source, the fungal culture was established by growth on glucose for 5 days, unless otherwise stated. On its depletion, coal was added to the medium and culturing continued.

Investigation of particle loading and size

The key operating variables of the slurry affecting coal biosolubilisation, namely coal loading and particle size were investigated using the factorial design detailed in Table 1. A 10 ml aliquot of the pre-inoculum culture was added into 150 ml media in 500 ml Erlenmeyer flasks and incubated on glucose for 5 days at 28°C with agitation at 120 rpm to establish the fungal culture. Where coal was used as carbon source, coal was added to the medium on glucose depletion and culturing continued for a further 14 days. The effect of particle size fraction was investigated across the extended range from 150-300 μm to 600-850 μm under shake flask conditions using an agitation rate of 120 rpm.

Sampling and Analytical Methods

Samples of 5 ml were taken aseptically at regular time intervals (8 sample total, equivalent to 40 ml). Biomass and coal were separated from the supernatant by vacuum filtration using glass-fibre filter membranes of 0.45 μm pore size. The supernatants were analysed for phenolic content, using the Folin-Ciocalteu reagent and spectroscopic analysis at 725 nm (17). Total organic carbon was quantified using an Anatoc Series II TOC analyser. Release of colour compounds into the supernatant was assessed by the absorbance at 450 nm. Coal weight loss was measured

gravimetrically after 14 days of coal solubilisation. Following removal of the supernatant, the spent biomass was washed off with water and the residual coal samples dried at 80°C for 48 hours. Solution or suspension pH was recorded every 48 hours. The microbial culture was observed microscopically at day 6.

Table 1: Experimental conditions for factorial experiment

Flask number	Coal loading (% w/v)	Particle size(μm)
1	5	600-850
2	10	600-850
3	5	1500-2000
4	10	1500-2000
5 Negative control (Un-Inoculated)	5	1500-2000
6 Positive control (No Coal)	-	-

Results and discussion

Products of coal biosolubilisation and their analysis

Coal biosolubilisation was quantified by measurement of the increase in absorbance of the supernatant at 450 nm owing to release of coloured compounds such as humic and fulvic acids. Further general release of organic materials into solution was observed by total organic carbon analysis (TOC). Potential for release of phenolic compounds was quantified by the Folin assay. Overall solubilisation was quantified by total mass loss of coal.

The product of coal biosolubilisation is reported to be a mixture of polar organic compounds with a high degree of aromaticity (6). Further, based on coal structure (18), release of phenolics and polyphenolics on solubilisation can be expected. Hofrichter *et al* (19) reported the production of 2-hydroxybiphenyl from the modification of hard coal (< 200μm) by *Coprinus sclerotigenus*. The release of other aromatic hydrocarbons, alkylated benzenes and branched alkanes were also reported but these compounds, probably present as part of the mobile phase, were liberated from micropores through the physical interaction with fungal hyphae. 4-Hydroxyphenylethanol, 1,2-benzendiol and 2- octenoic acid were obtained from the chloroform extract from coal degrading *T. atroviride* ES 11 reported recently (16). Contrary to this, Figure 1 shows no release of phenolics on incubation of coal with the fungus. The phenolic concentrations in the control test containing

the growth medium in the absence of coal were comparable to those measured in the presence of coal, and yielded a highest concentration of 97 mg l^{-1} . Hence the major source of phenolic compounds was the growth medium. Release of phenolic compounds on biosolubilisation of coal by *Trichoderma atroviride* ES11 was negligible.

Total organic carbon concentration (TOC) in solution provided a valuable quantification of the conversion of coal to soluble organic compounds. The change in TOC is given as a function of time during fungal coal biosolubilisation in Figure 2. On growth of fungi on the glucose media prior to coal addition over 5 days, the TOC decreased from 1800 mg l^{-1} to 250 mg l^{-1} (data not shown). After coal addition, the TOC increased to a concentration of 330 to 1000 mg l^{-1} over a 2 day period depending on the operating conditions. A sharp decrease in TOC was observed after the second day of coal biosolubilisation, owing to metabolism of these soluble products to CO_2 . Insignificant variation in TOC in coal slurries in the absence of fungal culture suggest that both the initial release of TOC between 0 to 2 days after coal addition and subsequent depletion of TOC resulted from microbial action rather than physical leaching.

The experimental set-up used in this study allowed the formation of microbial catalyst on glucose, followed by the assessment of its action on coal in the absence of glucose, unlike the set-up described in the work presented by Silva-Stenico *et al* (16), where catalyst production and coal biosolubilisation occur simultaneously. The latter system was more complex to analyse quantitatively. Qualitative GC-MS data showed a limited number of compounds at the end of the fermentation (11 days). In this study quantification of phenolic compounds and TOC showed these levels to be insignificant.

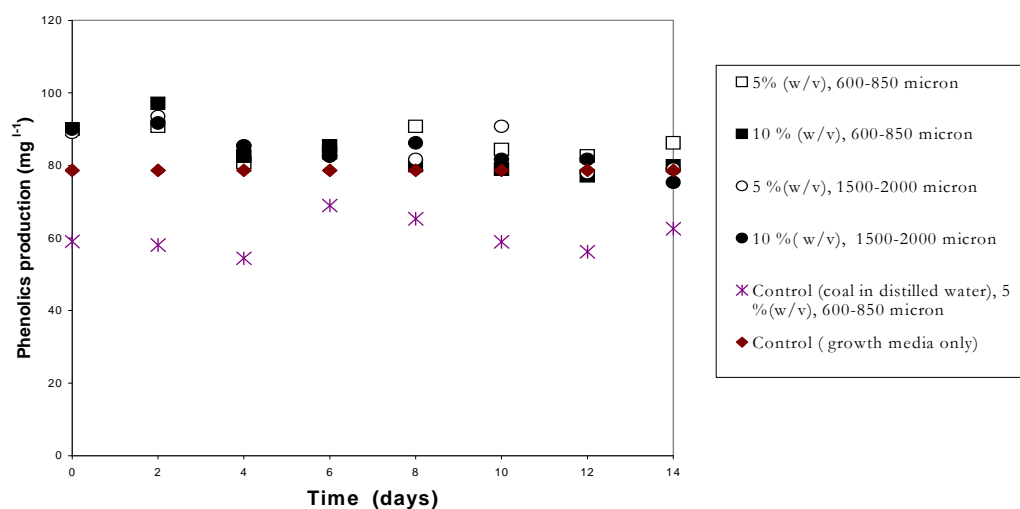


Figure 1: Phenolic production from *Trichoderma atroviride* ES 11 fungal strain biosolubilisation of coal in shake flask culture. Coal was added to a five-day *Trichoderma atroviride* culture on day 0.

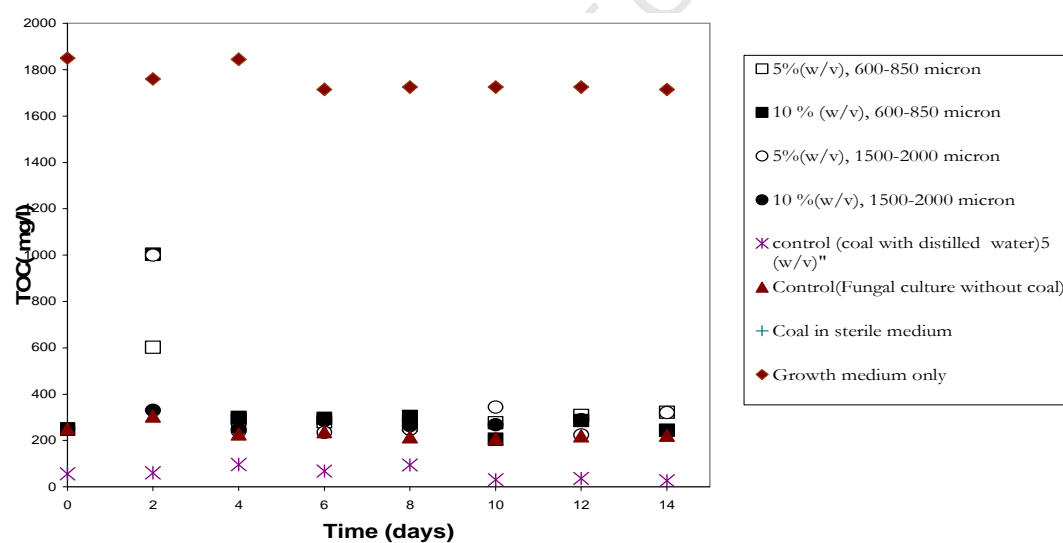
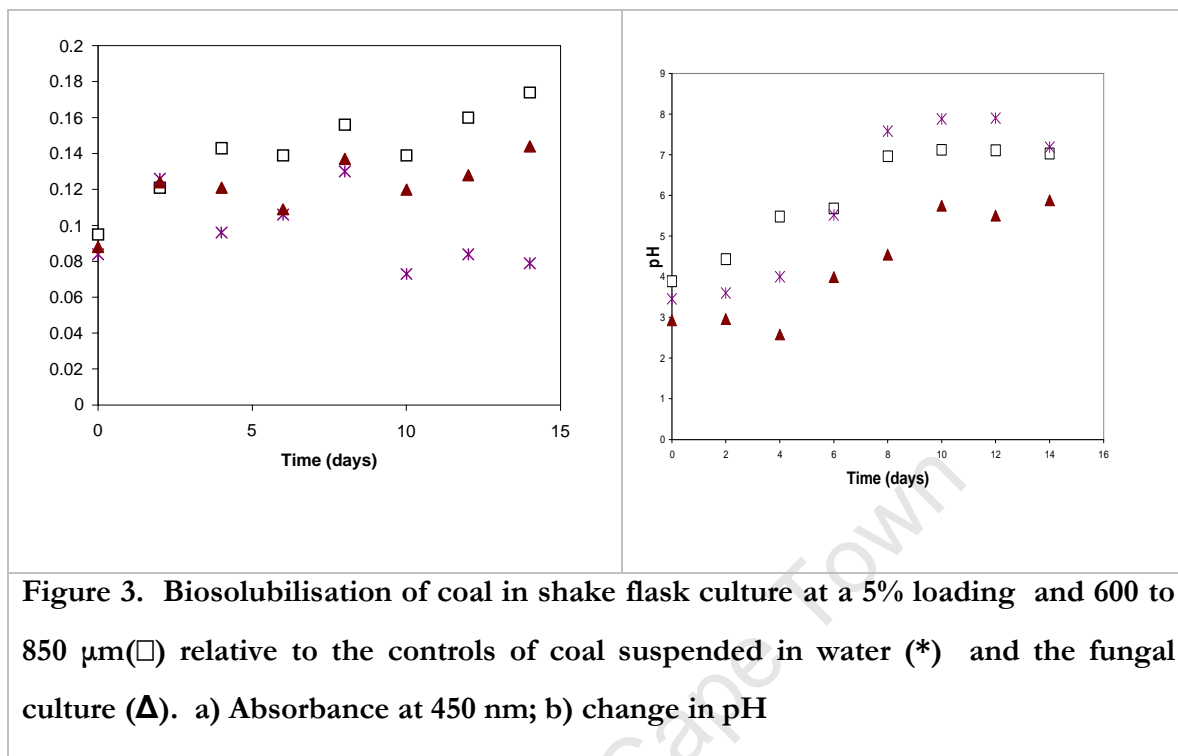


Figure 2: Total organic carbon concentration of fungal biosolubilisation of coal by *Trichoderma atroviride* in shake flask culture. Coal was added to a five-day *Trichoderma atroviride* culture on day 0.

In Figure 3a, the change in absorbance due to the release of colour compounds is presented, comparing the biosolubilisation of coal to fungal growth and abiotic coal suspension. While increase in A_{450} is observed on coal solubilisation, interference from the fungal culture occurred and the changes did not correlate quantitatively with coal solubilisation. Further, the release of

protons on reaction, shown as change in pH in Figure 3b, was not dominated by the bioreaction of coal.



Analysis of coal solubilisation

Critical and succinct assessment of the relative merits of the techniques used to evaluate the extent of coal solubilisation is given in Table 2. Simple spectrophotometric monitoring at an absorbance of 450 nm has been widely used to determine coal biosolubilisation; however, as shown in Figure 3, it is largely qualitative. The coal weight loss method is not widely used to determine coal solubilisation because it is tedious; however results from this study showed that it gave a reliable quantitative measurement of coal solubilisation. The negligible release of phenolic compounds during coal solubilisation (Figure 1) negates its use to determine the extent of coal solubilisation. The pH measurement shown in Figure 3b suggested that there were other dominant acid-consuming reactions interfering with coal solubilisation, hence pH measurement cannot be recommended as a method to determine the extent of coal solubilisation. Measurement of total organic carbon (TOC) provides potential for monitoring coal solubilisation and is useful to define the soluble components in the carbon balance; however these soluble compounds were further metabolised as shown in Figure 2, hence this method has limited use for monitoring solubilisation under these conditions.

Table 2: Analytical methods used in coal biosolubilisation

Parameters	Qualitative/ Quantitative	Interferences noted	Used in literature	Recommendation
Absorbance	Qualitative	No	Widely used	Yes
Weight loss	Quantitative	No	Not widely used	Yes
Phenolics		No release	Not used	No
TOC	Qualitative	Intermediate Metabolised	Not widely used	Yes
pH	Qualitative	Other dominant acid consuming reaction	Not widely used	No

Coal biosolubilisation as a function of particle size and loading

The effect of coal loading and particle size on coal solubilisation was investigated simultaneously through factorial analysis across the particle range 600-850 μm and 1500-2000 μm and the solids loading range 5% and 10%.

In Figure 4, absorbance at 450 nm is shown as a function of time under the experimental conditions given in Table 1. The absorbance did not increase in control tests containing growth medium only. An increase in absorbance occurred for both fungal culture with and without coal. The increase in absorbance was higher in the former. Hence, a correlation may exist between release of colour and coal solubilisation. The highest increase in absorbance occurred at 5 % (w/v) coal loading and 600 to 850 μm particle size fraction, suggesting that decreased particle size causes greater biosolubilisation.

An increase in coal loading of the same particle size fraction is expected to lead to an increase in absorbance owing to increased substrate availability. However, 5% (w/v) coal loading resulted in higher coal biosolubilisation in terms of colour increase than the 10% (w/v) case. This suggests that optimum coal loading may occur between 5 and 10% (w/v). Increase above this optimum may be postulated to lead to inhibition of the fungi involved in coal biosolubilisation owing to cell damage as reported by Nemati and Harrison (20) and Harrison *et al.* (21) for bacterial and yeast systems. This was confirmed by monitoring the microbial culture under both conditions microscopically (Figure 5) where fragmentation of the fungal mycelium was observed at 10% (w/v) loading.

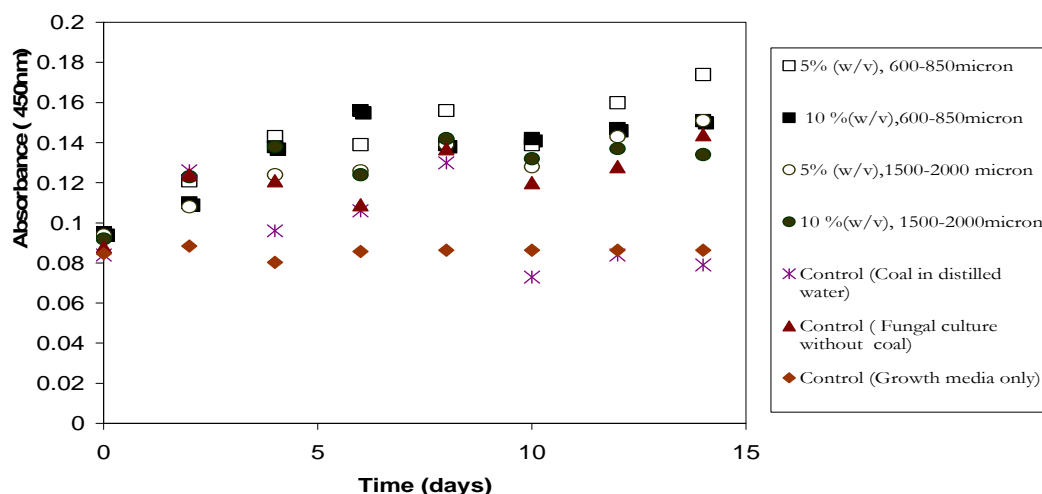


Figure 4 Biosolubilisation of coal by *Trichoderma atroviride* (ES 11) in shake flasks, measured in terms of colouration of the supernatant(A_{450}). The coal was added to a five-day *Trichoderma atroviride* culture on day 0 of the experiment.

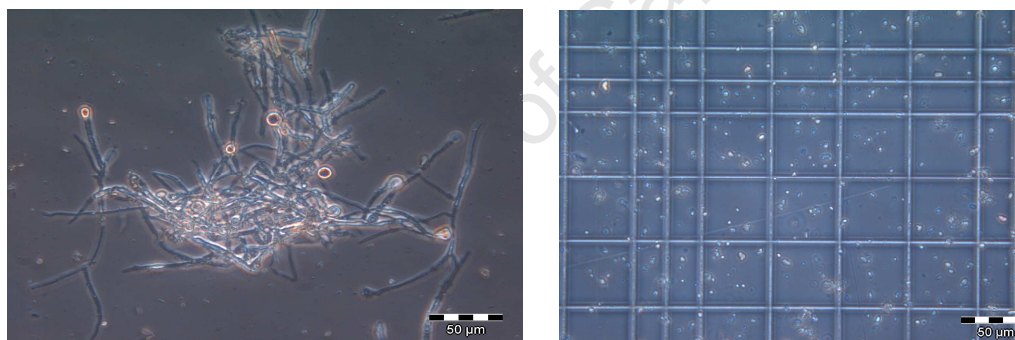


Figure 5: Micrographs of coal solubilisation at day 6 of shakeflask growth of *T. atroviride* with coal as carbon source at (A) 5%(w/v) and (B) 10%(w/v). Magnification 40x.

Reduction in coal weight has been used as a quantitative tool to determine coal solubilisation (15). Figure 6 shows coal weight loss following 14 day incubation with *Trichoderma atroviride* (ES11) as a percentage coal conversion. The smaller particle size resulted in higher coal weight loss in accordance with coal biosolubilisation being a surface phenomenon. The very low coal weight loss in the control experiments containing coal and distilled water confirmed that degradation of coal was mediated by the fungus or associated enzymes rather than a chemical leach reaction. In the factorial experiment, the highest degree of coal biosolubilisation of 9.3% by mass was found at 5% (w/v) and 600-850 μm .

There was an increase in pH in the fungal culture grown in the presence of coal (change in proton concentration of 238 mM), in the control where fungi were grown in the absence of coal (change in proton concentration of 578 mM) and the control where coal is incubated in distilled water (change in proton concentration of 330 mM). It is apparent that both biological and non-biological acid-consuming reactions occur. The consumption of protons under each experimental condition is presented in Table 3. The change observed in the presence of both coal and fungi was independent of coal loading, and not directly related to the extent of coal solubilisation. Hence the data presented here support the findings of Larboda *et al.* (10, 15) that variation in pH does not correlate with coal solubilisation.

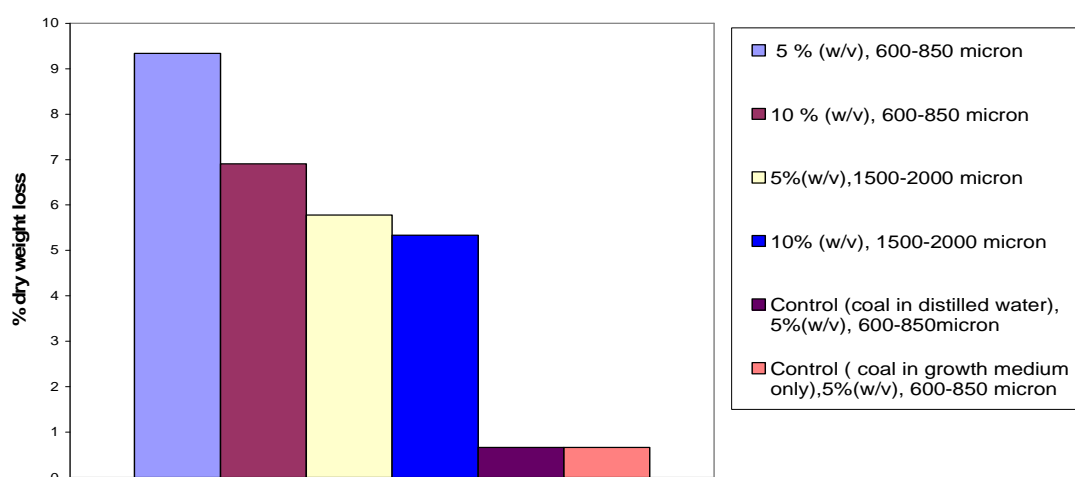


Figure 6: Coal biosolubilisation as a measure of decrease in percentage dry weight in relation to the initial coal weight. Factorial experiments were conducted in shake flasks. The coal was added to a five-day *Trichoderma atroviride* culture on day 0.

Table 3: Analysis of the change in pH in the factorial experiment.

Condition	Initial PH	Final pH	ΔH^+ (M)	Coal weight loss (%)
5%(w/v), 600-850 μ m	3.8	7.0	-0.0215	9.3
10%(w/v), 600-850 μ m	4.0	7.2	-0.0176	7.0
5%(w/v), 1500-2000 μ m	3.5	7.0	-0.0293	6.0
10%(w/v), 1500-2000 μ m	3.6	6.8	-0.0262	5.5
Coal only	3.4	7.8	-0.0330	0.5
Fungi only	2.8	5.8	-0.0578	

Analysis of factorial experiment of coal solubilisation

The use of a factorial experimental design allows a study of multi-parameter processes in which the dominant variables and interactions can be established. In addition, it aids the search for optimum conditions (22). In this study, a two-level factorial design was employed to investigate the parameters of coal loading and particle size fraction. In terms of the measured response, gravimetric weight loss was used since it provided a quantitative measure of coal biosolubilisation.

Yates' procedure provides a simple method of analysing a 2^n factorial experiment to provide a simple linear model:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 \quad \text{Equation 1}$$

where Y is the measured response, x_i is the variable i. The significance of algorithm is that it facilitates the determination of the algebraic signs of the coefficients needed for calculating the main and interaction effects of each factor in a factorial experiment. Each factor is coded with a low level denoted -1 and the high level denoted +1.

The coal weight loss data and factorial combination are presented in Table 4. The estimate of the effect of each variable and their interaction was computed by using the Yates' algorithm, shown in Table 5.

Table 4: Coal weight loss by factorial analysis. Run 1 & 2: independent replicates.

Factor		Coal loading	Initial wt	% Coal weight loss (Run 1)	% Coal weight loss (Run 2)	% Total coal weight loss
Size a	Loading b					
-1	-1	5%, 600-850 μ m	7.5	9.3	18.76	28.09
+1	-1	10%, 600-850 μ m	15	7.0	12.77	19.67
-1	+1	5%, 1500-2000 μ m	7.5	5.78	5.16	12.28
+1	+1	10%, 1500-2000 μ m	15	5.3	5.26	11.12

Table 5: Effect estimate and sum of squares estimated using Yates method of statistical analysis

Combination	Total Response	(1)*	(2)#	Effect estimate	Sum of Squares (SS)
(1)	28.09	47.76	71.23	Total	188
a	19.67	23.46	-9.53	-4.77	22.7
b	12.28	-8.43	-24.30	-12.15	148
ab	11.12	-1.10	7.32	3.66	10.4

1)* - The first entry in column (1)* represents the sum of the response of the first two entries in response column (i.e (1)+a). The second entry is the sum of the second two entries in the response column (i.e b and ab). The third entry is the difference between the first two entry responses. (i.e a-(1)). The fourth entry is the difference between the second pair of responses (ab-b).

(2)# -The same procedure is applied using data in column (1)*

An empirical relationship was developed from the factorial analysis to provide a prediction of coal biosolubilisation as percentage weight loss (Y) in terms of particle size fraction (b) and coal loading (a):

$$Y = \frac{1}{2} \langle 17.79 + (-2.83)A + (-6.08) + (1.83)AB \rangle \quad \text{Equation 2}$$

where 17.79 is the average response and A and B can be +1 or -1. Equation 2 can be decoded to the actual values of particle size and coal loading reported in Table 4, as shown in Equation 3:

$$Y = 8.895 - 1.19 \left[\frac{a - 0.5(10 + 5)}{0.5(10 - 5)} \right] - 3.04 \left[\frac{b - 0.5(1.75 + 0.725)}{0.5(1.75 - 0.725)} \right] + 0.915 \left[\frac{a - 0.5(10 + 5)}{0.5(10 - 5)} \right] \left[\frac{b - 0.5(1.75 + 0.725)}{0.5(1.75 - 0.725)} \right] \quad \text{Equation 3}$$

Further simplification gives

$$Y = 26.43 - 1.38a - 11.28b + 0.713ab \quad \text{Equation 4}$$

where **a** is % loading and **b** is nominal diameter (mm)

Equation 4 suggests that the particle size fraction, coal loading and the interaction between particle size and coal loading affect degradation of coal. The coefficient in the linear model shows that particle size fraction (b) is the most important variable, followed by the coal loading and the interaction between variables.

Extending the operating window of particle size

Owing to the dominant effect of size, it was desirable to extend the operating window of particulate size considered. Experiments were carried out at 600-850 µm and 150-300 µm at 5 % (w/v) coal loading. The effect of particle size on coal solubilisation is presented in Figure 7. Results from both qualitative (release of coloured compounds measured as absorbance at 450 nm) and quantitative (decrease in dry mass of coal) analyses of coal biosolubilisation show that extent of solubilisation of the 150-300 µm particle size fraction and the resultant accumulation of coloured products in solution from this fraction was increased. The extent of coal degradation was increased by 4-fold when using a size fraction of 150-300 µm in comparison with the 600-850 µm fraction. Coal biosolubilisation is a surface phenomenon, demonstrated by obtaining better biosolubilisation when a smaller size fraction is used (12). In Table 6, coal solubilisation is

presented as a function of nominal particle surface area (assuming spherical particles). The extent of coal mass loss across 150-300 μm with a specific surface area of $2.17 \text{ cm}^2 \text{ g}^{-1}$ was 28 % compared with a 7.8% at 600-850 μm ($0.54 \text{ cm}^2 \text{ g}^{-1}$) and a 5.6% at 1500-2000 μm ($0.21 \text{ cm}^2 \text{ g}^{-1}$).

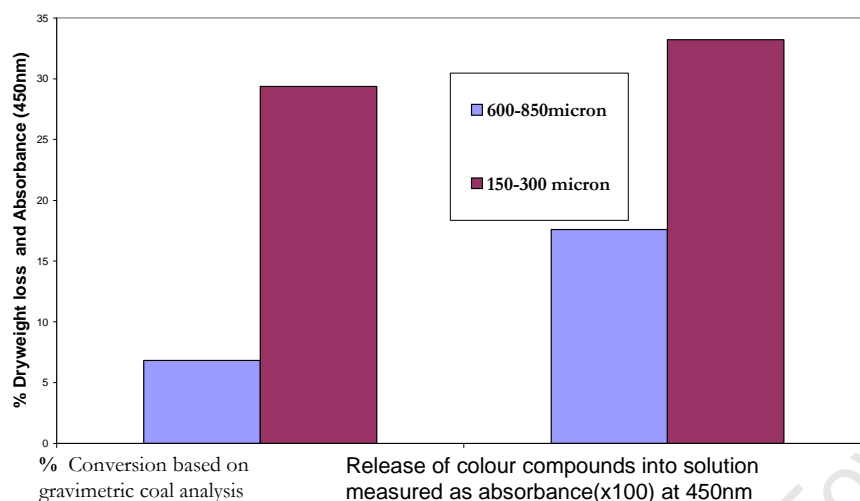


Figure 7: Effect of particle size fraction on coal solubilisation during coal biosolubilisation with *Trichoderma atroviride* ES11

Table 6: Effect of particle size fraction and available surface area on coal biosolubilisation

Particle size fraction	Initial coal	Mass solubilised	Specific surface area ($\text{cm}^2 \text{ g}^{-1}$)	% Coal weight loss
150-300 μm	7.5	2.5	$2.17 \text{ cm}^2 \text{ g}^{-1}$	28
600-850 μm	7.5	0.6	$0.54 \text{ cm}^2 \text{ g}^{-1}$	7.8
1500-2000 μm	7.5	0.375	$0.21 \text{ cm}^2 \text{ g}^{-1}$	5.6

Conclusion

This study has shown that coal weight loss and colour absorbance can be used to monitor coal biosolubilisation. Particle size fraction, coal loading and their interaction affect the coal biosolubilisation process. Under the conditions studied in the factorial experiment (loadings of 5 to 10 %, size fraction of 600 to 2000 μm), the highest degree of coal biosolubilisation was at 5% (w/v) coal loading using a particle size fraction of 600-850 μm . Mechanical damage of the fungi was observed at 10% (w/v) coal loading. Coal biosolubilisation was enhanced by increased availability of surface area achieved either by increased loading (up to the critical value) or reduced particle size. Decreasing the particle size fraction from 600-850 μm to 150-300 μm resulted in a 4-fold increase in surface area with an associated increase in the degree of coal solubilisation of 4-fold. A 28% coal solubilisation was observed with a particle size fraction of 150-300 μm at 5% coal loading.

No significant increase in phenolics concentration was observed on coal biosolubilisation. Further, the TOC analysis suggested that, following release of soluble organic compounds from coal, these are further metabolised to CO_2 since no significant increase in TOC was maintained through the experiment.

Acknowledgements

The authors gratefully acknowledge the National Research Foundation of South Africa and the University of Cape Town for providing financial support for the work presented.

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